Growth and development of larval bay scallops (*Argopecten irradians*) in response to early exposure to high CO_2

by

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Abstract

Coastal and estuarine environments experience large variability and rapid shifts in pCO_2 levels. Elevated pCO_2, or ocean acidification, often negatively affects early life stages of calcifying marine invertebrates, including bivalves, but it is unclear which developmental stage is most sensitive. I hypothesized that initial calcification is a critical stage during which high pCO_2 exposure has severe effects on larval growth and development of bay scallop (*Argopecten irradians*). Using five experiments varying the timing of exposure of embryonic and larval bay scallops to high CO_2, this thesis identifies two distinct stages of development during which exposure to high CO_2/low pH causes different effects on bay scallop larvae. I show that any exposure to high CO_2 consistently reduces survival of bay scallop larvae. I also show that high CO_2 exposure during initial calcification (12-24 h post-fertilization) results in significantly smaller shells, relative to ambient conditions, and this size decrease persists through the first week of development. High CO_2 exposure at 2-12 h post-fertilization (pre-calcification), does not impact shell size, suggesting that the CO_2 impact on size is a consequence of water chemistry during calcification. However, high CO_2 exposure prior to shell formation (2-12 h post-fertilization) causes a high incidence of larval shell deformity, regardless of CO_2 conditions during initial calcification. This impact does not occur in response to high CO_2 exposure after the 2-12 h period. The observations of two critical stages in early development has implications for both field and hatchery populations. If field populations were able to time their spawning to occur during the night, larvae would undergo initial calcification during the daytime, when CO_2 conditions are more favorable, resulting in larger veliger larvae. Hatcheries could invest minimal resources to monitor and modify water chemistry only during the first day of development to ensure larva are exposed to favorable conditions during that critical period.

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This thesis is dedicated to my Poppa:

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Contents

Chapter 1

Introduction

1.1 Ocean Acidification

Since the industrial revolution, the burning of fossil fuels, cement production, and land-use changes have increased atmospheric concentrations of carbon dioxide (CO_2) from preindustrial levels of approximately 280 ppm to the approximately 390.5 ppm today (Conway and Tans 2012). The rate of this increase is an order of magnitude higher than has occurred for millions of years (Doney and Schimel 2007). Over time, much of this CO_2 moves from the atmosphere into the ocean, where it reduces seawater pH. Within the past decade, this process, known as ocean acidification (OA), has gained recognition in part because it is likely to have an impact on both calcifying and non-calcifying marine organisms.

1.2 Ocean Carbonate System

Once CO_2 enters the ocean, its chemistry is governed by a series of chemical reactions:

$$CO_{2(g)} \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + H^+ \quad (1)$$

Dissolved CO_2 reacts with water to form carbonic acid (H_2CO_3), which further dissociates to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) and two hydrogen ions. These reactions are reversible and approach equilibrium rapidly (seconds to minutes) in seawater (Zeebe and Wolf-Gladrow 2005). The sum of the concentrations of dissolved CO_2, carbonic acid, bicarbonate, and carbonate ions makes up the total dissolved inorganic carbon (DIC). At the pH of seawater (~8.1), 90 % of DIC is in the form of bicarbonate, 9 % is in the form of carbonate, and 1 % is

in the form of dissolved CO_2. When CO_2 is added to the system, it increases the total DIC as well as the concentration of hydrogen ions, which results in a decrease in pH. As pH drops, it shifts the equilibrium between carbonate species, increasing bicarbonate concentration and decreasing carbonate concentration (Stumm and Morgan 1996).

The decrease in carbonate ion concentration has implications for biocalcification because it reduces the saturation state of calcium carbonate. Calcium carbonate dissolves to form carbonate and calcium ions in a dissolution reaction governed by the solubility product K_{sp}:

$$CaCO_3 \xleftrightarrow{K_{sp}} CO_3^{2-} + Ca^{2+} \quad (2)$$

Calcium carbonate formation and precipitation vary with calcium carbonate saturation state (Ω), defined as:

$$\Omega = \frac{[Ca^{2+}]_{observed}[CO_3^{2-}]_{observed}}{K_{sp}} \quad (3)$$

Saturation state depends on temperature, pressure, salinity, and the mineral phase of calcium carbonate, with aragonite being more soluble than calcite. When Ω is greater than 1, seawater is supersaturated and precipitation of calcium carbonate and biocalcification is thermodynamically favorable. When Ω is less than 1, seawater is undersaturated, or corrosive, and pure calcium carbonate and unprotected shells will begin to dissolve. Calcium ion concentration is proportional to salinity, so when salinity variations are small, such as in the open ocean, Ω is largely determined by changes in carbonate ion concentration (Stumm and Morgan

1996; Zeebe and Wolf-Gladrow 2005). When carbonate ion concentration decreases as a result of decreasing pH, Ω also decreases. A drop in Ω can make calcification (or shell-building) more difficult, or more energetically costly for calcifying marine organisms (Orr et al. 2005; Gazeau et al. 2007; Waldbusser et al. 2011).

1.3 Coastal and Estuarine Acidification

The mechanisms by which increased pCO_2 affects pH and Ω are the same, regardless of how the CO_2 enters the system. However, the processes by which CO_2 enters the water differ between open ocean systems and coastal and estuarine systems. In the open ocean, the majority of CO_2 enters the water from the atmosphere through air-sea exchange. Coastal and estuarine environments, where many commercially and economically important organisms live, experience larger variability and more rapid shifts of pCO_2 levels than the open ocean (Cai and Wang 1998; Howarth et al. 2011; Hofmann et al. 2011). Carbonate chemistry fluctuates as a result of both natural and anthropogenic processes on timescales ranging from daily to seasonal (Melzner et al. 2012). During daytime, photosynthesis accounts for a decrease in pCO_2 and an increase in dissolved oxygen (DO). At night, photosynthesis ceases and respiration of autotrophs and heterotrophs alike results in increased pCO_2 and a decrease in DO. This creates an approximately 24-h cycle of higher pH during the daytime and lower pH at night. These cycles can be easily seen in the data provided by the National Estuarine Research Reserve System's Centralized Data Management Office (NOAA 2012). For example, at Childs River in

the Waquoit Bay National Estuarine Research Reserve, DO and pH are measured *in situ* every 15 minutes throughout the year; in July 2008, DO and pH reached peak values in the late afternoon/early evening (~3:00 – 6:00 pm) and minimum values in the morning (~5:00 – 8:00 am; NOAA 2004). Seasonally, microbial respiration of organic matter increases during summer months when the water temperature increases. Along with seasonal stratification, this can lead to sub-surface areas of increased CO_2 and decreased pH and DO (Dai et al. 2006; Diaz and Rosenberg 2008; Howarth et al. 2011; Melzner et al. 2012). Eutrophication of coastal and estuarine waters further expands the seasonal variability of dissolved CO_2 in coastal and estuarine systems by fueling massive algal blooms and therefore increasing the amount of organic matter available for microbial respiration (Rabalais et al. 2002; Galloway et al. 2008; Diaz and Rosenberg 2008; Cai et al. 2011).

In addition to the impacts of seasonal cycles and eutrophication on their carbonate chemistry, coastal and estuarine environments are also affected by global OA resulting in increased atmospheric CO_2 levels (Caldeira and Wickett 2003; Doney et al. 2009; Feely et al. 2009). Coastal regions may be more vulnerable to OA than open ocean regions because their buffering capacity is reduced relative to the open ocean (Zeebe and Wolf-Gladrow 2005; Denman et al. 2007; Feely et al. 2009; Borges and Gypens 2010; Cai et al. 2011; Melzner et al. 2012; Sunda and Cai 2012). Therefore, it is possible that increases in CO_2 could have a greater effect on pH in coastal and estuarine systems than on open ocean systems (Cai et al. 2011; Melzner et al. 2012).

1.4 Exposure of Marine Larvae to Coastal and Estuarine Acidification

Early life stages of marine invertebrates are particularly vulnerable to a variety of environmental conditions including both chemical and physical stressors (Pechenik 1987), and their development is key for the successful recruitment, and therefore survival, of the species (Cowen et al. 2000). There is increasing evidence that early life stages of marine invertebrates, particularly of those that produce calcareous skeletons or shells, are negatively affected by high CO_2 conditions. Many calcifying marine invertebrates, including bivalves, spawn during summer months (Belding 1910; Costello and Henley 1971) when pCO_2 levels are at the highest and Ω is the lowest (Feely et al. 2010; Waldbusser et al. 2011; Melzner et al. 2012). As atmospheric CO_2-driven OA and anthropogenic eutrophication increase, the conditions experienced by coastal and estuarine bivalve larvae will become increasingly unfavorable for shell growth and development. Additionally, all bivalves, including those that produce calcite shells as adults, produce their larval shells from aragonite, a more soluble form of calcium carbonate (Carriker and Palmer 1979), which could increase the vulnerability of bivalve larvae to high CO_2 conditions.

Some bivalve species appear to be more tolerant of OA conditions than others (Miller et al. 2009), but negative effects of OA have been shown on all early life stages of bivalves, including fertilization, larval development, and juvenile development (Parker et al. 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al.

2010; Parker et al. 2010; Waldbusser et al. 2011; Gazeau et al. 2011; Van Colen et al. 2012). The pH decrease since pre-industrial times may have already had an impact on bivalve larvae, as both hard clam (*Mercenaria mercenaria*) and bay scallop (*Argopecten irradians*) larvae experienced delayed metamorphosis when exposed to present-day atmospheric CO_2 conditions relative pre-industrial conditions (Talmage and Gobler 2010). Exposure to high CO_2/low pH water has been shown to decrease survival, delay metamorphosis, and negatively affect growth of bivalve larvae (Watson et al. 2009; Van Colen et al. 2012).

While the evidence is clear that larval bivalves are strongly impacted by high CO_2 conditions, it is not well understood how variable exposure to high CO_2 conditions affects larval growth, development, and survival, nor at what stage is such exposure most critical. Such questions have relevance to both natural ecology and practical applications. In the field, larvae are exposed to variable CO_2 conditions spatially as they are transported through estuaries and temporally on daily timescales as described above. Also, larvae of many bivalve species are reared in commercial hatcheries (Shumway and Parsons 2006), and hatcheries would benefit from an understanding of which developmental stages are most vulnerable to high CO_2 conditions and how exposure at different stages may affect larval development differently.

1.5 Thesis Goals

I conducted experiments to determine how exposure to high CO_2 (~2200 ppm) during the earliest stages of embryonic and larval development affect bay scallop (*Argopecten irradians*) larval growth, survival, and development. The experiments were also designed to determine if there is a period of early development in which exposure to high CO_2 has critical, persistent impacts on later development, and to identify the timing of that period as closely as possible. Embryonic and larval bay scallops were exposed to high CO_2 conditions for variable amounts of time at varying stages of their development. Each experiment included initial and subsequent CO_2 exposures (i.e, a "switch" design, Fig. 1.1), with the timing of the transition as the main difference among experiments. Most experiments consisted of four experimental treatments with four different CO_2 exposure regimes: continuous exposure to ambient CO_2 (ambient treatment), continuous exposure to high CO_2 (high CO_2 treatment), initial exposure to ambient CO_2 followed by exposure to high CO_2 (switch to high CO_2 treatment), and initial exposure to high CO_2 followed by exposure to ambient CO_2 (switch to ambient treatment). Four different experiments were performed in which the switch occurred 2 h post-fertilization (Fertilization Experiments 1 and 2), 12 h post-fertilization (12-h Switch Experiment), 24 h post-fertilization (24-h Switch Experiment), or 3 d post-fertilization (3-d Switch Experiment). The timings of these switches were chosen based on the timing of events during early bay scallop larval development (Fig. 1.2).

Each chapter presents the results of 1 or 2 different experiments. In Chapter 2, the 3-d Switch Experiment shows that exposure to high CO_2 during early larval

development negatively impacts larval size and that switching the CO_2 conditions after the first three days of development does not affect larval size. In Chapter 3, the results of Fertilization Experiments 1 and 2, indicate that exposure to high CO_2 during fertilization affects survival, but not larval size. In Chapter 4, the results of the 12- and 24-h Switch Experiments show that exposure to high CO_2 conditions prior to calcification produces different effects on larval development than exposure to high CO_2 conditions during initial calcification.

The combined results of all the experiments show that any exposure to high CO_2 conditions consistently reduce survival of larval bay scallops. I also find that exposure to high CO_2 during the onset of calcification (12-24 h post-fertilization) negatively impacts larval size of bay scallops and that exposure to high CO_2 prior to shell formation (2-12 h post-fertilization) causes a high incidence of larval shell deformity, regardless of CO_2 conditions during initial calcification. These results have both ecological and commercial implications. Based on daily fluctuations in CO_2 conditions, the timing of scallop spawning in the field could result in the larvae being exposed to either favorable or unfavorable conditions during the critical 12-24 h post-fertilization period. Additionally, based on my findings, hatcheries could limit their monitoring and modification of water chemistry to during the first day of development only. Because this first day of development is critical to larval size and shell formation, but exposure to high CO_2 after this period does not affect either aspect, hatcheries would not need to invest resources into water chemistry manipulation beyond the first day.

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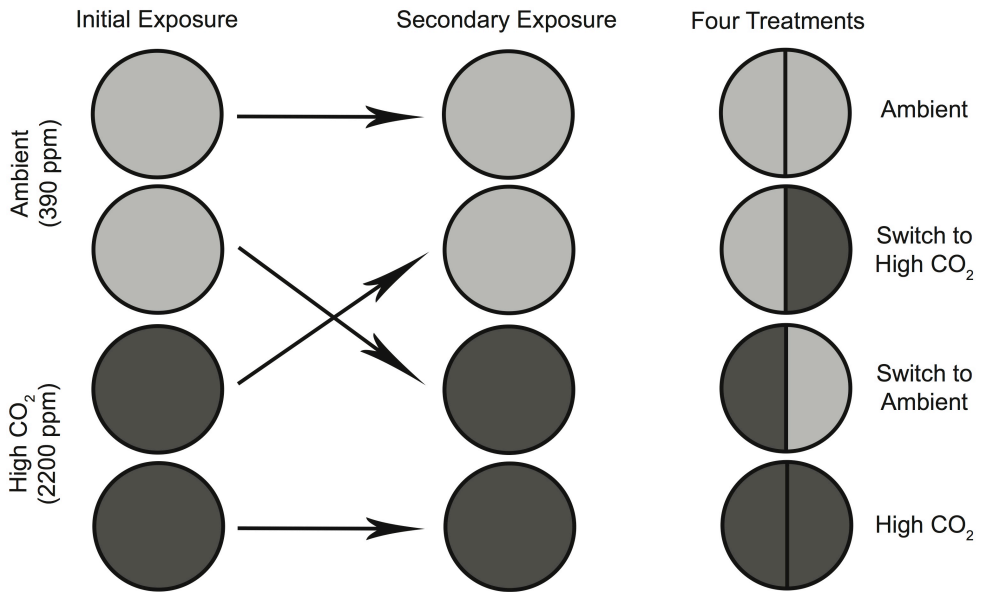

Figure 1.1. Schematic of the experimental design used for all experiments described in this thesis. Scallop larvae were initially exposed to either ambient or high CO_2 conditions for a period of time. Subsequently, half of the scallops exposed to each CO_2 condition were switched to the other CO_2 condition, resulting in four treatment groups, each with a different CO_2 exposure regime. The main difference between the experiments is the age at which the switch in CO_2 conditions occurred.

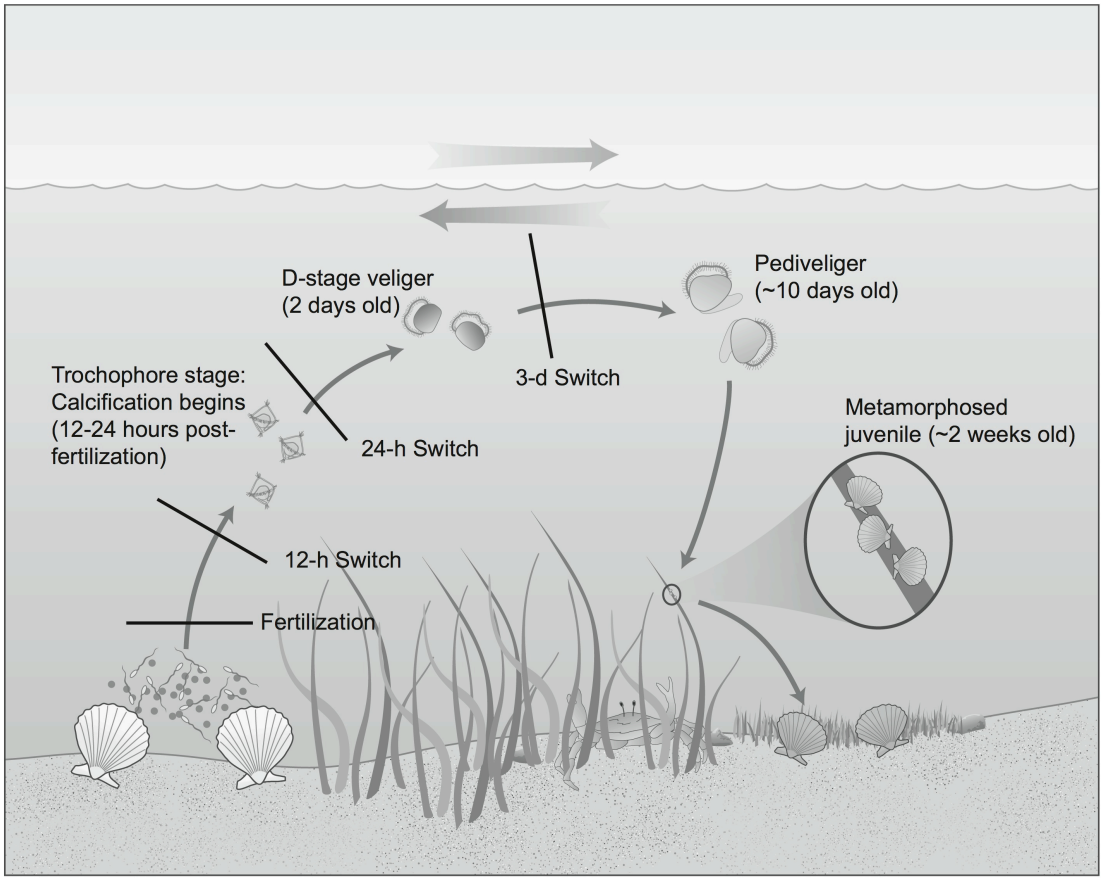

Figure 1.2. Bay scallop life cycle showing the timing of the switch in each experiment. Bay scallop adults live on the seafloor, but larvae spend their time in the water column and are transported by tidal and wind-driven currents. During the trochophore stage (~ 12 h post-fertilization), the larval shell starts formation and calcification. By the time larvae are 1 d old, the shell is generally fully calcified. When larvae are 2 d old, they have reached the D-stage, with a shell that looks like a capital letter 'D.' At about 10 d, the larvae develop a foot and are known as pediveligers. They test substrates to find a location ideal for settling. Larvae generally metamorphose to juveniles by the time they are 2 wk old. Juveniles may live on a substrate such as eelgrass, but when they grow larger, they move to the seafloor and live there for up to 2 yr, although the majority are harvested by commercial fishermen when they are 1.5 yr old.

Chapter 2

Early exposure of bay scallops (*Argopecten irradians*) to high CO_2 causes a decrease in shell growth

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2.1 **Abstract**

Ocean acidification, characterized by elevated pCO_2 and the associated decreases in seawater pH and calcium carbonate saturation state (Ω), has a variable impact on the growth and survival of marine invertebrates. Larval stages are thought to be particularly vulnerable to environmental stressors, and negative impacts of ocean acidification have been seen on fertilization as well as on embryonic, larval, and juvenile development and growth of bivalve molluscs. We investigated the effects of high CO_2 exposure (resulting in pH = 7.39, Ω_{ar} = 0.74) on the larvae of the bay scallop *Argopecten irradians* from 12 h to 7 d old, including a switch from high CO_2 to ambient CO_2 conditions (pH = 7.93, Ω_{ar} = 2.26) after 3 d, to assess the possibility of persistent effects of early exposure. The survival of larvae in the high CO_2 treatment was consistently lower than the survival of larvae in ambient conditions, and was already significantly lower at 1 d. Likewise, the shell length of larvae in the high CO_2 treatment was significantly smaller than larvae in the ambient conditions throughout the experiment and by 7 d, was reduced by 11.5 %. This study also demonstrates that the size effects of short-term exposure to high CO_2 are still detectable after 7 d of larval development; the shells of larvae exposed to high CO_2 for the first 3 d of development and subsequently exposed to ambient CO_2 were not significantly different in size at 3 and 7 d than the shells of larvae exposed to high CO_2 throughout the experiment.

2.2 Introduction

Coastal marine invertebrates are exposed to dissolved carbon dioxide levels that fluctuate on time scales ranging from daily to seasonal as a result of both natural processes and human activities (Cai and Wang 1998; Howarth et al. 2011; Hofmann et al. 2011). These aqueous CO_2 levels are likely to increase (and pH to drop) in the decades ahead as a consequence of ocean acidification (OA), the uptake of anthropogenic CO_2 by the ocean (Feely et al. 2004; Orr et al. 2005; Doney et al. 2009). Since pre-industrial times, increases in anthropogenic emissions of CO_2 have caused a decrease in surface ocean water pH of 0.1 units, and a decrease of another 0.2-0.3 units is projected by the end of this century (Orr et al. 2005; Feely et al. 2009). As seawater pH decreases, its calcium carbonate saturation state (Ω) also decreases, and drops in Ω have the potential to make calcification (or shell-building) more difficult, or more energetically costly for the organism (Orr et al. 2005; Gazeau et al. 2007; Waldbusser et al. 2011).

While changes in surface ocean pH are happening on a global scale, the processes affecting coastal and estuarine ocean pH are different and result in more seasonably variable conditions than in the open ocean (Feely et al. 2010; Howarth et al. 2011; Cai et al. 2011). Anthropogenic eutrophication is a major factor influencing acidification of coastal and estuarine regions (Borges and Gypens 2010; Waldbusser et al. 2011; Sunda and Cai 2012). Eutrophication results in algal blooms, which eventually die, sink to the seafloor, and fuel microbial respiration (Rabalais et al. 2002; Diaz and Rosenberg 2008). This process is exacerbated during summer

stratification events (Chou et al. 2009) and can produce low-pH seasonal bottom waters that are undersaturated with respect to aragonite (Feely et al. 2010). Because many coastal bivalve species spawn during summer months, the larvae are exposed to such conditions. Furthermore, because the buffering capacity of seawater is reduced as dissolved inorganic carbon (DIC) increases, it has been suggested that eutrophication could increase the susceptibility of coastal waters to ocean acidification (Cai et al. 2011). As atmospheric CO_2-driven OA and anthropogenic eutrophication increase, the conditions experienced by bivalve larvae will become increasingly unfavorable for shell growth.

Marine invertebrate larvae are vulnerable to a variety of environmental conditions including both chemical and physical stressors (Pechenik 1987). There is mounting evidence for negative effects of OA on marine invertebrate larvae, especially bivalve mollusc species that produce calcareous skeletons or shells. For example, mussel and oyster larvae raised in water with pH ~7.4 were shown to have delayed development to D-stage veligers compared to larvae raised in ambient pH ~8.1 (Kurihara et al. 2007; 2008). Talmage and Gobler (2010) showed that hard clams (*Mercenaria mercenaria*) and, to a lesser extent, bay scallops (*Argopecten irradians*) experienced delayed metamorphosis at present-day conditions compared to pre-industrial conditions, suggesting that the 0.1 pH decrease in the last 150 years has already had an impact on bivalve species. OA has been shown to have a negative impact on multiple early life stages of bivalves, including fertilization, D-stage (early) development, later larval development, and juvenile development

(Parker et al. 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al. 2010; Van Colen et al. 2010; Parker et al. 2010; Waldbusser et al. 2011; Gazeau et al. 2011). Some species appear to be more tolerant of OA conditions than others. For example, two species of oysters displayed different trends when exposed to a range of pCO_2 values resulting in an aragonite saturation state ($\Omega_{aragonite}$) of 1.3 – 0.6 for ~30 days; *Crassostrea virginica* had decreased shell area and decreased calcification with decreasing Ω_{ar}, but *Crassostrea ariakensis* showed no change in shell area or calcification (Miller et al. 2009). Nonetheless, the majority of work has demonstrated that young bivalves are negatively impacted by the high CO_2/low pH conditions resulting from ocean acidification. There is evidence that the earliest larvae of some species are susceptible to OA (Kurihara et al. 2007; 2008; Van Colen et al. 2012). Additionally, OA conditions have been shown to negatively affect the survival of bay scallop larvae and the size of competent and post-metamorphic bay scallops (Talmage and Gobler 2009; 2010; 2011).

Here, we address the impact of early exposure to elevated CO_2 on the survival and growth of bay scallop larvae. Bay scallops are ideal as a model organism for this study because of their economic importance as a commercially harvested shellfish and because of their relatively short larval duration (~2-3 weeks). We also investigate whether transferring the larvae to ambient conditions can reverse the effects of early high CO_2 exposure. Such a scenario is ecologically relevant in a situation where larvae are spawned in an estuary with relatively high pCO_2 and are subsequently transported by currents or tides out of the estuary to sites with a

lower pCO_2. We exposed larvae to ambient (nominally 390 ppm CO_2, pH = 7.93) and high (nominally 2200 ppm CO_2, pH= 7.39) CO_2 conditions for a total of 18 days. The high CO_2 treatment produced a calcium carbonate saturation state that was undersaturated with respect to aragonite. Such pCO_2 values and associated saturation states have been observed in summer months in a local estuary (Childs River, Falmouth, MA, USA) where bivalve larvae are found (McCorkle et al. 2012). In addition, we exposed a third group of larvae to high CO_2 conditions for three days (through the larval D-stage), followed by exposure to ambient CO_2 conditions for 15 days. We show that larvae exposed continuously to high CO_2 have significantly smaller shells at 1 d, relative to larvae exposed continuously to ambient CO_2 and that this difference in size persists throughout the first week of development. We also show that a switch to ambient CO_2 conditions after initial 3 d exposure to high CO_2 does not affect larval shell size.

2.3 Methods

2.3.1 Adult Collection and Spawning

Adult *A. irradians* (subspecies *irradians*) individuals were collected during spring and summer months from coastal waters around Mashpee, Massachusetts and were held in submerged cages in Little River, an estuarine river near Waquoit Bay, Massachusetts, until needed. Several days prior to spawning, the adults were brought to Woods Hole Oceanographic Institution, where they were maintained in

16 °C flowing seawater and fed daily with Instant Algae Shellfish Diet (Reed Mariculture, Campbell, CA, USA).

Spawning was induced by placing the hermaphroditic adults in a 20 °C bath with ambient CO_2 flowing seawater and gradually raising the temperature to a maximum of 25 °C. When an individual spawned, it was moved to a beaker of 20 °C, 0.35 μm filtered seawater (FSW) and the spawned gametes were examined to distinguish eggs from sperm. To prevent self-fertilization, the water in the beakers was changed every 10-15 min. Eggs were rinsed through a 75 μm filter to remove debris, collected on a 20 μm filter, and subsequently pooled. Seawater with sperm was rinsed through a 20 μm filter to remove debris and the sperm was pooled. Eggs were collected from four individuals and sperm was collected from ten individuals. If a scallop released both eggs and sperm, only the eggs were used. Sperm and eggs were each pooled separately into about 1 L of seawater. About 1 ml of sperm was added to the eggs and the embryos were left to develop in the beaker for 11 h, until the larvae were at the swimming gastrula stage (Belding 1910).

2.3.2 Larval Culture

When the scallop larvae were 11 h post-fertilization, they were homogeneously suspended in the beaker by gentle plunging with a graduated cylinder (Helm and Bourne 2004) and 1 ml was removed to estimate their density. Live larvae were counted at 100X magnification on a gridded slide in which each grid square held 1 μl (Widman et al. 2001).

Larvae were stocked at an initial density of 30 larvae ml^{-1} and were maintained in 800 ml of 0.35 µm FSW in six 1-l polyethylene cups per treatment, which were previously conditioned in running seawater for at least four weeks. Cultures were fed daily with laboratory-raised *Isochrysis galbana* (Tahitian strain, T-iso) in the exponential phase of growth at a density of 37,500 cell ml^{-1}. This ration has been shown to produce good growth rates and survivorship of bay scallop larvae (Widman et al. 2001). Culture water was changed every three days with pre-CO_2 equilibrated FSW. During water changes, each culture was gently poured through a 20 µm sieve, which caught the larvae. The larvae were rinsed back into the cup and the cup was filled to 800 ml. To maintain a stable temperature, all culture cups were contained in a water bath controlled by an aquarium chiller/heater (T = 22.5 ± 0.3 °C).

2.3.3 Manipulation of Water Chemistry

Water chemistry was manipulated by bubbling cultures with either compressed air or a mixture of compressed air and pure CO_2. The ambient treatment was bubbled with compressed air produced by an oil-free, portable air compressor (Porter Cable, Jackson, TN, USA). The high-CO_2 treatment was bubbled with a compressed air/pure CO_2 mixture precisely controlled using two mass flow controllers (Aalborg, Orangeburg, NY, USA). To create the high CO_2 treatment, 8.1 ml min^{-1} CO_2 was mixed with 4.5 l min^{-1} compressed air. Each 800 ml culture cup was bubbled at a rate of approximately 100 ml min^{-1}.

Filtered seawater was pre-CO_2 equilibrated by bubbling with the appropriate air-CO_2 mixture in 14-l buckets for 24 h prior to filling the 1 l culture cups. Each of the ambient and high-CO_2 treatment replicate cups was bubbled with ambient CO_2 compressed air or high CO_2-compressed air mixture, respectively, for the 18-d duration of the experiment. The high CO_2 to ambient switch treatment (hereafter referred to as the switch-to-ambient treatment) was bubbled with the high CO_2-compressed air mixture for the first three days of the experiment, followed by ambient CO_2 compressed air for the remaining 15 d of the experiment.

2.3.4 Characterization of Water Chemistry

Prior to water changes, the carbonate chemistry of the pre-equilibrated water in the 14-l buckets was measured. To characterize the carbonate chemistry of the water, pH, total alkalinity, salinity, and temperature were measured. Spectrophotometric pH measurements were made with 2 mM *m*-Cresol purple indicator dye to ensure high accuracy and precision using an Ocean Optics USB4000 Spectrometer with an LS-1 light source and a FIA-Z-SMA-PEEK 100 mm flow cell (Ocean Optics, Dunedin, FL, USA), following the procedure described by Clayton and Byrne (1993) and Dickson et al. (2007), and using the refit equation of Liu et al. (2011). This method proved to have a precision of ±0.002 pH units.

Samples for total alkalinity analysis were filtered to 0.45 μm, poisoned with saturated mercuric chloride, and stored in sealed glass vials until analysis. Total alkalinities were measured in duplicate via titration with 0.01 M HCl using a

Metrohm Titrando 808 and 730 Sample Changer controlled by Tiamo software to perform automated Gran titrations of 1 ml samples. Laboratory (or "in house") seawater standards were calibrated using seawater certified reference materials (supplied by the laboratory of Andrew Dickson, Scripps Institution of Oceanography) and were included in each run. Gran titrations had a precision of ± 2 μEq kg^{-1}.

Salinity was determined using a Guildline model 8400B "Autosal" laboratory salinometer (Guildline Instruments, Smith Falls, Ontario, Canada). The temperature of the water bath was recorded every 10 min by a TidbiT v2 data logger (Onset Computer Corporation, Pocasset, MA, USA) and was also recorded for each culture cup at the time of pH measurements.

Based on the measured values of pH (seawater scale), total alkalinity, temperature, and salinity, we used CO2SYS Software (Pierrot et al. 2006) to calculate pCO_2, $\Omega_{aragonite}$, and total DIC using the first and second dissociation constants (K_1 and K_2) of carbonic acid in seawater from Mehrbach et al. (1973), refit by Dickson and Millero (1987).

The high-CO_2 treatment produced carbonate chemistry conditions (Table 2.1) comparable to those seen in Waquoit Bay during summer months (McCorkle et al. 2012) when many bivalve species, including bay scallops, spawn.

2.3.5 Microscopic Imaging and Shell Measurements

At 1, 3, and 7 d, approximately 50 larvae from each culture were preserved in 95 % ethanol for subsequent microscopic imaging and shell measurement. At the time of imaging, the preserved larvae were transferred to FSW and viewed at 200X magnification under bright field transmitted light using a Nikon ECLIPSE 50i POL microscope. Images were captured using a SPOT Insight™ Camera controlled by SPOT Basic Software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Using the built-in measurement capabilities of the software program, shell length (the longest dimension parallel to the hinge) was measured for at least 15 larvae from each culture. Mean growth rate (μm d^{-1}) for each replicate was calculated as the increase in mean shell length from 1 d to 7 d, divided by the number of days (6 d).

2.3.6 Survival Estimation

Percent survival was estimated at 1, 3, 7, and 18 d. By 18 d, less than 0.5 % of larvae remained alive in any treatment, so both survival and shell size analyses focused on 1, 3, and 7 d. On these days each culture was homogeneously suspended by gently plunging with a graduated cylinder, and a known volume (13, 25, and 40 ml for 1, 3, and 7 d, respectively) was removed and concentrated by gently pouring it through a 20 μm sieve. The volumes were chosen to yield a number of larvae (100-200) that could be counted in approximately 30-45 min. All of the live larvae in this volume were counted under a stereomicroscope at about 10X magnification. The density of larvae in the removed volume was used to calculate the percent survival based on the initial stocking density of 30 larvae ml^{-1}. One replicate culture

in the ambient treatment was discovered to have unreliable survival counts, as a result of a mistake by the counter, so only data from the other five replicate cultures were analyzed.

2.3.7 Statistical Analysis

All statistical analyses were performed using Systat® 13 Software (Systat Software, Inc., Chicago, IL, USA). Percent survival data were arcsine-square root-transformed prior to statistical analyses. Repeated measures ANOVA tests were run to compare survival among the three treatments at 1, 3, and 7 d and to compare shell length among the three treatments at 1, 3, and 7 d. One-way ANOVAs followed by Tukey's Honestly Significant Difference tests were run to compare survival and shell length among the three treatments at each time-point separately. A one-way ANOVA was run to compare growth rates from day 1-7 among the three treatments.

2.4 Results

2.4.1 Shell Length

Larval development (Fig. 2.1) in all treatments progressed in a sequence typical for this species as described by Belding (1910) and Widman et al. (2001). All larvae were fully shelled at 1 d and were post-D-stage by 3 d. While fully shelled, some 1 d larvae had velums that protruded from the shell (Fig. 2.1D, G); this

occurred more frequently in larvae from the switch-to-ambient and high-CO_2 treatments than in larvae from the ambient treatment.

Exposure to high CO_2 (Table 2.1) caused a significant reduction in shell length (Fig. 2.2, Tables 2.2, 2.3) during the first week of development when compared to exposure to ambient CO_2 (repeated measures ANOVA, Wilk's Lambda = 0.033; $F = 19.47$; df = 6, 26; $p < 0.00001$). This pattern was significant for each of days 1, 3, and 7 (One-way ANOVA; Table 2.3). On day 1, after 12 h of exposure, the mean shell lengths of all three treatments were significantly different from each other (Fig. 2.2). The difference on day 1 between the switch-to-ambient and high-CO_2 treatments was unexpected, as the larvae were in similar conditions prior to the switch on day 3. On days 3 and 7, the mean shell lengths of larvae from the high-CO_2 and switch-to-ambient treatments were no longer significantly different from each other, despite having been in different conditions since day 3. The mean lengths of shells from the high-CO_2 treatment were 84.1 %, 92.5 %, and 88.5 % of the mean lengths of shells from the Ambient CO_2 treatment on days 1, 3, and 7, respectively. After day 1, mean shell growth rates (Table 2.2) integrated over following 6 days were not significantly different among any treatments (one-way ANOVA, $F = 0.50$, df = 2, $p = 0.62$).

2.4.2 Larval Survival

Survival of scallop larvae in the ambient treatment was consistently higher than survival of scallop larvae in either the switch-to-ambient or high-CO_2

treatments (Fig. 2.3) during the first week of development. This overall effect on survival was significant in a repeated measures ANOVA (Wilk's Lambda = 0.38; F = 2.50; df = 6, 24; p = 0.05). However, when the effect was examined for individual days, survival in the ambient treatment was significantly higher only on day 1 (Table 2.4, Fig. 2.3). Survival of larvae in the switch-to-ambient treatment was not significantly different than in the other treatments at any time during the first week of development.

2.5 Discussion

Early exposure (12-24 hours post-fertilization) to high CO_2 significantly reduced larval shell size (Fig. 2.2, Table 2.2) and survival (Fig. 2.3) relative to ambient CO_2 by the time the larvae were 1 d old. The initial reduction in size relative to the ambient treatment was still evident after the first week of larval development. This suggests that CO_2 exposure during the first day is critical to shell development. Growth rate from 1-7 d was not significantly affected by CO_2 exposure, further indicating that growth during the first 24 h post-fertilization determines shell size later in development – the larvae did not increase their growth rate to compensate for initial slow growth. There is some evidence that bivalve larvae use amorphous calcium carbonate (ACC) to produce the earliest stages of their shell (Weiss et al. 2002). If this is true, then it may be a factor in explaining the sensitivity of bay scallop larvae to high CO_2 conditions during the first day of development, as ACC is more soluble than aragonite and its formation would

therefore be less thermodynamically favorable. This demonstration of a significant and lasting CO_2 effect on shell size within the first day of larval development suggests that other studies on bivalve larval development in which CO_2 exposure was initiated after the first day of larval development and initial calcification (Talmage and Gobler 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al. 2010; Talmage and Gobler 2010; Van Colen et al. 2012) may have underestimated the magnitude of the effects of high CO_2 throughout larval development.

Exposure to high CO_2 caused a significant decrease, relative to ambient conditions, in larval survival at 1 d post fertilization, and a consistent, but not significant, decrease at 3 and 7 d post fertilization. Survival of < 20 % of individuals on day 7 is low compared to other studies of this species (Talmage and Gobler 2009; 2010), probably due to a combination of not using antibiotics, and calculating survival from initial counts at 12 h post-fertilization rather than 3 d. Mortality during the first 3 d of larval development typically is high and can be variable between culture vessels even when conditions are held constant (Widman, J.C. Jr., personal communication). In our study, survival was highly variable among replicates within treatments and was likely influenced by factors other than carbonate chemistry. This variability may contribute to unexplainable patterns in survival such as the differences on day 1 between high-CO_2 and switch-to-ambient treatments, despite the similar conditions.

A size reduction in scallop larvae exposed to high CO_2 may have indirect effects on subsequent survival in the field. The age at which a scallop larva is

competent to metamorphose is often affected by size (Sastry 1965). Small scallop larvae may delay metamorphosis, increasing their time in the plankton and risk of mortality from planktonic predators (Thorson 1950). Previous work has found that exposure of bivalve larvae to high CO_2 treatments leads to delayed metamorphosis, although it is not clear if small size was the cause of the delay (Talmage and Gobler 2009; 2011; Van Colen et al. 2012). In addition, if the smaller size of bay scallop larvae exposed to high CO_2 persisted through metamorphosis into adulthood, the reproductive output of those individuals could be stunted simply because the size of the gonad is proportional to the size of the scallop (Barber and Blake 2006).

The negative effect of high CO_2 exposure on bay scallop larval size is consistent with negative effects of high CO_2 exposure on the size of other larval bivalves. The clam *Macoma balthica* has been shown to produce significantly smaller larval shells at 3 d when exposed to seawater with a pH of 7.5 or 7.8 directly after fertilization, compared to a control treatment with pH 8.1 (Van Colen et al. 2012). The same group also reported a similar trend in which *M. balthica* larvae produced (non-significantly) smaller shells from day 5 to 19 when exposed to lowered pH (Van Colen et al. 2012). Similarly, larvae of the blue mussel, *Mytilus edulis*, had shells that, at 2 d, were 12.7 % smaller when raised in water with pH 7.6 compared to larvae grown in water with pH 8.1 (Gazeau et al. 2010). Larvae of the oyster *Saccostrea glomerata* showed at 8 d a decrease in shell size of 6.3 % at pH 7.8 and 8.7 % at pH 7.6 relative to pH 8.1 (Watson et al. 2009). By documenting a size effect in the early larval stage of bay scallops, we can better understand previously

published observations of negative effects of high CO_2 exposure on shell size of 19-20 d old competent scallops (Talmage and Gobler 2009; 2010; 2011). Our results suggest that a decrease in size of competent larvae and set juveniles exposed to high CO_2 may be a lingering effect of compromised growth in early stages.

Acidification of coastal waters is affected by atmospheric CO_2 levels, but it is also strongly impacted by eutrophication (Feely et al. 2010; Howarth et al. 2011; Cai et al. 2011; Sunda and Cai 2012), mixing and circulation (Gilbert et al. 2010; Feely et al. 2010), and input of fresh water (Cai et al. 2011; Sunda and Cai 2012). We showed a CO_2 exposure effect on larval bay scallop growth, but we did not include hypoxia as a treatment. Hypoxia typically co-occurs with high CO_2 (hypercapnia) as a result of eutrophication in coastal and estuarine waters (Dai et al. 2006; Howarth et al. 2011; Sunda and Cai 2012). Because hypoxia and hypercapnia act synergistically on the responses of marine invertebrates found in such environments (Widdicombe and Spicer 2008), it is possible that the interaction of low oxygen and high CO_2 will affect larval bay scallop development even more strongly than high CO_2 alone.

The larvae in both high-CO_2 and ambient treatments maintained similar growth rates from 1-7 d, but the negative effects on shell length of exposure to high CO_2 during the first 3 d of development were still present after a week of larval development. This result suggests that there is a critical initial window in which CO_2 exposure is particularly damaging to scallop larvae. Larvae that spawn in coastal and estuarine environments when pCO_2 is high may have smaller shells,

even if they are transported within a few days to waters with lower CO_2. Aquaculture facilities may need to monitor pCO_2 in the water used for rearing early larvae to ensure that conditions do not impair growth.

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Table 2.1. Environmental parameters and carbonate chemistry for the Ambient and High CO_2 treatments during the experiment (mean ± SD). Calculated parameters were calculated from pH and total alkalinity using CO2SYS software (Pierrot et al. 2006) with K_1, K_2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), $KHSO_4$ from Dickson (1990), and pH measured on the seawater scale. (A_T = total alkalinity; $[HCO_3^-]$ = bicarbonate ion concentration; $[CO_3^{2-}]$ = carbonate ion concentration; $[CO_2]$ = dissolved carbon dioxide concentration; DIC = dissolved inorganic carbon; $\Omega_{aragonite}$ = aragonite saturation state.)

	Ambient CO_2	**High CO_2**
Measured parameters		
Temperature (°C)	22.4 ± 0.3	22.5 ± 0.2
Salinity	32.2 ± 1.1	31.8 ± 0.7
pH	7.93 ± 0.01	7.39 ± 0.03
A_T (µEq kg^{-1})	2130 ± 68	2114 ± 44
Calculated parameters		
pCO_2 (µatm)	509 ± 13	1987 ± 140
$[HCO_3^-]$ (µmol kg^{-1})	1779 ± 46	2000 ± 44
$[CO_3^{2-}]$ (µmol kg^{-1})	142 ± 10	46 ± 3
$[CO_2]$ (µmol kg^{-1})	16 ± 1	61 ± 4
DIC (µmol kg^{-1})	1937 ± 55	2107 ± 47
$\Omega_{aragonite}$	2.26 ± 0.14	0.74 ± 0.04

Table 2.2. Mean (± SD) size (µm) at 1, 3, and 7 d and mean (± SD) growth rate (µm d^{-1}) from 1-7 d of A*rgopecten irradians* larvae raised in three CO_2 treatment regimes; *n* = 6.

Treatment	Day 1	Day 3	Day 7	Growth Rate
Ambient	87.4 ± 0.4	96.6 ± 0.4	131.8 ± 9.2	7.4 ± 1.5
Switch to Ambient	78.0 ± 2.1	90.1 ± 1.1	118.2 ± 8.1	6.7 ± 1.2
High CO_2	73.5 ± 1.9	89.4 ± 2.6	116.7 ± 6.5	7.2 ± 1.0

Table 2.3. One-way ANOVAs of mean shell length (µm) of *Argopecten irradians* larvae raised in three CO_2 treatment regimes (ambient, switch-to-ambient, and high-CO_2) at 1, 3, and 7 d; n = 6.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
Day 1	Treatment	610.774	2	305.387	114.244	<0.001
	Error	40.097	15	2.673		
Day 3	Treatment	187.631	2	93.816	35.058	<0.001
	Error	40.140	15	2.676		
Day 7	Treatment	823.314	2	411.657	6.382	0.010
	Error	967.488	15	64.499		

Table 2.4. One-way ANOVAs of mean percent survival (arcsine-square root-transformed) of *Argopecten irradians* larvae raised in three CO_2 treatment regimes (ambient, switch-to-ambient, and high-CO_2) at 1, 3, and 7 d; n = 5 for ambient treatment, n = 6 for switch-to-ambient and high-CO_2 treatments.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
Day 1	Treatment	0.040	2	0.020	3.731	0.050
	Error	0.074	14	0.005		
Day 3	Treatment	0.004	2	0.002	0.502	0.616
	Error	0.056	14	0.004		
Day 7	Treatment	0.012	2	0.006	1.694	0.219
	Error	0.051	14	0.004		

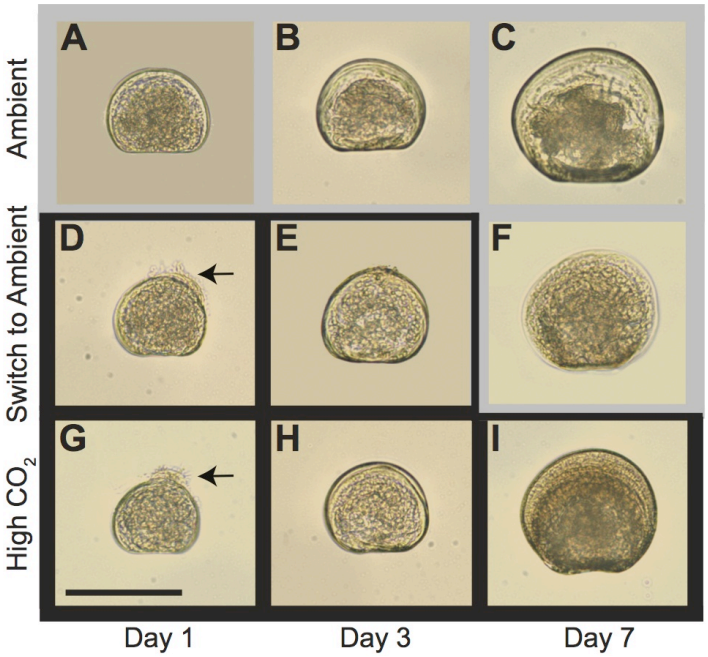

Figure 2.1. Larval morphology of bay scallops (*Argopecten irradians*) exposed to varied pCO_2 conditions. Larvae were preserved in 95 % ethanol after incubation for 1 d, 3 d, and 7 d in one of the three CO_2 treatment regimes (ambient, switch-to-ambient, and high-CO_2). Larvae shown represent the mean shell length for each treatment and age. The outline of each image corresponds to the CO_2 treatment the larvae were experiencing at the time of preservation. Gray = ambient CO_2, black = high CO_2. Arrows indicate exposed velum. Images are all to the same scale; scale bar = 100 μm.

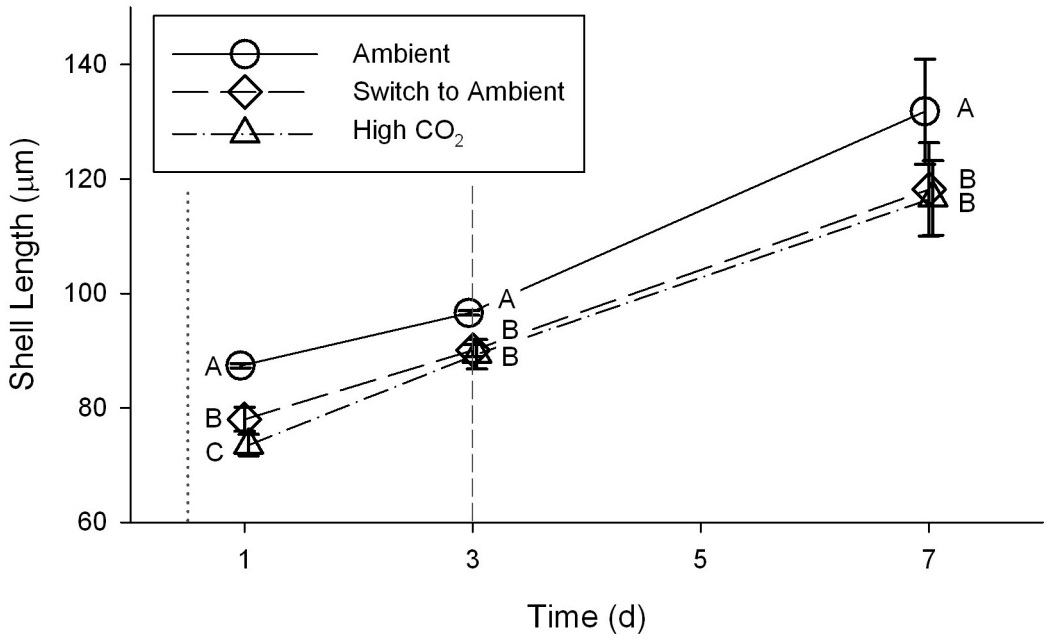

Figure 2.2. Shell length of larval bay scallops (*Argopecten irradians*) during the first week of larval development. Values are mean ± SD of 6 replicate culture containers. The dotted vertical line indicates the age at the time of inoculation (exposure to CO_2 treatments); the dashed vertical line indicates the age at which CO_2 conditions were switched for the switch-to-ambient treatment. Different letters (A, B, C) denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 3), followed by Tukey's HSD test.

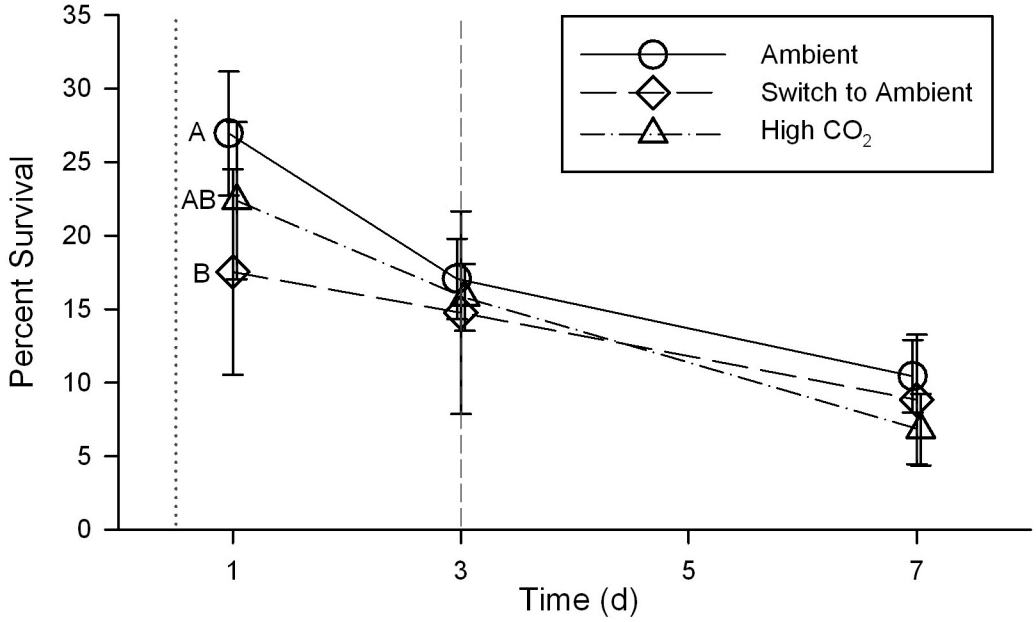

Figure 2.3. Survival of larval bay scallops (*Argopecten irradians*), expressed as the percent of larvae surviving from the time of inoculation (age = 0.5 d), during the first week of larval development. Values are mean ± SD of $n = 5$ replicate culture containers for the ambient treatment and $n = 6$ replicate culture containers for the switch-to-ambient and high-CO_2 treatments. The dotted vertical line indicates the age at the time of inoculation (exposure to CO_2 treatments); the dashed vertical line indicates the age at which CO_2 conditions were switched for the switch-to-ambient treatment. Letters denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 4), followed by Tukey's HSD test; only the ambient and high-CO_2 treatments on day 1 are significantly different.

Chapter 3

Elevated pCO_2 during fertilization of the bay scallop *Argopecten irradians* reduces larval survival but not shell size

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In preparation

3.1 Abstract

Ocean acidification (OA), characterized by elevated pCO_2, generally has negative effects on early life stages of invertebrates. However, it is unclear which stage might be most sensitive to exposure to high CO_2 condtions. We investigated the idea that fertilization is a critical stage for the bay scallop *Argopecten irradians* during which exposure to elevated pCO_2 (resulting in pH = 7.28, Ω_{ar} = 0.63) has severe and persistent effects, relative to ambient CO_2 conditions (pH = 7.96, Ω_{ar} = 2.52). We investigated the effects of exposure to high CO_2 on the *A. irradians* from fertilization to 7 d old. To assess the possibility of persistent effects of fertilization-exposure, further treatments included switches from high CO_2 to ambient CO_2 and from ambient CO_2 to high CO_2 just after fertilization. Survival of larvae that had been fertilized in high CO_2 conditions was significantly lower than the survival of larvae fertilized in ambient conditions. In contrast, CO_2 conditions during fertilization did not affect larval shell size; rather larvae that developed post-fertilization in high CO_2 had significantly smaller shells than larvae that developed post-fertilization in ambient CO_2 regardless of in which conditions they were fertilized.

3.2 Introduction

The ocean and the atmosphere naturally exchange great amounts of carbon dioxide (CO_2), but as human activities, including as fossil fuel burning, increase the atmospheric concentration of CO_2, the ocean is forced to take up an increasing amount of CO_2 (Le Quéré et al. 2009). Once in the ocean, dissolved CO_2 goes through a series of reactions which release H^+ ions, decreasing the pH of the water through a process known as ocean acidification (OA) (Feely et al. 2004; Orr et al. 2005; Doney et al. 2009). In the past 200 years, increases in anthropogenic emissions of CO_2 have caused a decrease in surface ocean water pH of 0.1 units, and a decrease of another 0.2-0.3 units is projected by the end of this century as a result of increasing atmospheric CO_2 (Orr et al. 2005; Doney et al. 2009). In addition to lowering seawater pH, OA also results in a decrease in calcium carbonate saturation state (Ω), which has the potential to make calcification (shell-building) more difficult, or more energetically costly for calcifying organisms (Orr et al. 2005; Gazeau et al. 2007; Waldbusser et al. 2011).

While OA is affecting the surface ocean through uptake of atmospheric CO_2 on a global scale, the processes affecting pH and Ω in coastal and estuarine environments are more complex and more extreme (Feely et al. 2010; Howarth et al. 2011; Cai et al. 2011). Water chemistry in coastal and estuarine environments is influenced by many factors including freshwater inputs, eutrophication, respiration, and stratification (Borges and Gypens 2010; Waldbusser et al. 2011; Cai et al. 2011; Melzner et al. 2012; Sunda and Cai 2012). For example, when algal blooms resulting

from eutrophication die and sink to the seafloor, they fuel microbial respiration, which releases CO_2 (Rabalais et al. 2002; Diaz and Rosenberg 2008). Stratification during summer months isolates the CO_2 released from microbial respiration (Chou et al. 2009) and can result in bottom waters that are undersaturated with respect to calcium carbonate (Feely et al. 2010). Models have shown that the carbonate chemistry of coastal environments is more strongly influenced by eutrophication than by atmospheric CO_2-driven OA (Borges and Gypens 2010) and that at current atmospheric CO_2 levels, seawater can easily become severely undersaturated ($\Omega <$ 0.3) during periods of eutrophication (Melzner et al. 2012).

Marine invertebrates living in coastal and estuarine environments are exposed to dissolved carbon dioxide levels that fluctuate on time scales ranging from daily to seasonal as a result of both natural processes and human activities (Cai and Wang 1998; Howarth et al. 2011; Hofmann et al. 2011). Many coastal bivalve species spawn during summer months, when O_2 and CO_2 conditions tend to be least favorable as a result of stratified waters and increased microbial respiration. The coinciding timing of unfavorable conditions and spawning is a serious issue for marine invertebrates because early life stages are particularly vulnerable to both chemical and physical environmental stressors (Pechenik 1987), including OA, but are also key for successful recruitment and survival of the species (Cowen et al. 2000).

An increasing amount of work has shown negative effects of high CO_2 conditions on early life stages of multiple species of marine invertebrates, especially

bivalve species that produce calcareous shells. Some bivalve species appear to be more tolerant of high CO_2 conditions than others (Miller et al. 2009), but various work has shown negative effects of high CO_2 on all early life stages of bivalves, including fertilization, larval development, and juvenile development (Parker et al. 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al. 2010; Parker et al. 2010; Waldbusser et al. 2011; Gazeau et al. 2011; Van Colen et al. 2012). For example, Talmage and Gobler (2010) showed that hard clams (*Mercenaria mercenaria*) and bay scallops (*Argopecten irradians*) experienced delayed metamorphosis at present-day CO_2 conditions relative to pre-industrial conditions, suggesting that the pH decrease since pre-industrial times has already had an impact on bivalve larvae. Blue mussels (*Mytilus edulis*) were shown to have significantly smaller shells at 2 d old when exposed to water with pH 7.6, relative to water with pH 8.1, and to have significantly thinner shells at 15 d old when exposed to water with pH 7.8, relative to water with pH 8.0 (Gazeau et al. 2010). In addition to developmental delays and stunted growth, decreases in survival of larval bivalves have also been noted; Watson et al. (2009) found that survival of oyster larvae (*Saccostrea glomerata*) raised in pH 7.6 was reduced by 72 % relative to larvae raised in pH 8.1.

Most previous bivalve studies introduced larvae to high CO_2 conditions after embryonic development, or even after larval development, has begun, leaving open the question of effects during fertilization. The few studies that have examined high CO_2 effects during fertilization have produced mixed results. Both the Sydney rock oyster (*S. glomerata*) and the Pacific oyster (*Crassostrea gigas*) had reduced

fertilization success and impaired larval development when exposed to 1000 ppm CO_2 and suboptimal temperatures, relative to exposure to 375 ppm CO_2 at optimal temperature (Parker et al. 2009; 2010). In contrast, Havenhand and Schlegel (2009) found no significant difference in fertilization success in *C. gigas* at pH 8.15 and pH 7.8, nor did they find a difference in sperm mobility or swimming speed. A decrease in fertilization success of the clam *Macoma balthica* was seen when gametes were fertilized at pH ~7.5 and ~7.8, compared to pH 8.1 (Van Colen et al. 2012). Previous studies have not examined how exposure to elevated CO_2 during fertilization affects bivalve larval survival and development beyond 3 days of age, nor have they investigated whether negative effects are reversible.

In order to investigate these ideas, we designed a culturing experiment where adults were induced to spawn in either ambient or high CO_2 conditions and the resulting larvae were raised for one week. Additionally, half of the embryos from each fertilization group had their CO_2 exposure switched 2 h post-fertilization to determine whether any negative effects of high CO_2 exposure during fertilization could be reversed by subsequent ambient exposure. For the high CO_2 exposure, we used 2200 ppm CO_2 , resulting in $pH_{(seawater\ scale)}$ = 7.28 and a calcium carbonate saturation state that was undersaturated with respect to aragonite ($\Omega_{aragonite}$). Such pCO_2 values and associated saturation states have been observed in summer months in a local estuary (Childs River, Falmouth, MA, USA) where bivalve larvae are found (McCorkle et al. 2012). The results of these culturing experiments showed that exposure of bay scallop gametes to high CO_2 prior to and during fertilization

reduced larval survival. In contrast, we found that high CO_2 exposure during fertilization did not affect larval shell size; rather high CO_2 exposure during intial calcification (post-fertilization) determined larval shell size.

3.3 Methods

3.3.1 Manipulation of Water Chemistry

Water chemistry was manipulated as described in section 2.3.3 of Chapter 2. The high CO_2 treatment (~2200 ppm) produced carbonate chemistry conditions (Table 3.1) comparable to those seen in Waquoit Bay during summer months (McCorkle et al. 2012) when many bivalve species, including bay scallops, spawn.

3.3.2 Characterization of Water Chemistry

To characterize the carbonate chemistry of the treatment water, pH, total alkalinity, salinity, and temperature were measured following the procedures described in section 2.3.4 of Chapter 2. Briefly, pH was measured spectrophotometrically following the procedure described by Clayton and Byrne (1993) and Dickson et al. (2007), and using the refit equation of Liu et al. (2011). Alkalinity was measured by Gran titration with 0.01 M HCl. Based on the measured values of pH (seawater scale), total alkalinity, temperature, and salinity, CO2SYS Software (Pierrot et al. 2006) was used to calculate pCO_2, $\Omega_{aragonite}$, and total DIC

using the first and second dissociation constants (K_1 and K_2) of carbonic acid in seawater from Mehrbach et al. (1973), refit by Dickson and Millero (1987).

In addition to the replicate culture cups with larvae, 2 cups, one for each CO_2 level, were maintained without larvae or algae added to them to serve as abiotic references for the chemistry characterization. The FSW in these cups was analyzed for carbonate chemistry and changed every 2 d, as for the replicate culture cups.

3.3.3 Adult Collection

Adult *A. irradians* individuals were collected during winter and spring months from coastal waters around Martha's Vineyard and Woods Hole, Massachusetts and were held in submerged cages in Little River, a estuarine river near Waquoit Bay, Massachusetts until needed. Scallops were collected under a research collection permit issued by the Commonwealth of Massachusetts Department of Fish and Game, Division of Marine Fisheries. Several days prior to spawning, the adults were brought to Woods Hole Oceanographic Institution, where they were maintained in 16 °C flowing seawater and fed daily with Instant Algae Shellfish Diet (Reed Mariculture, Campbell, CA, USA).

3.3.4 Control of Spawning Conditions

Spawning was induced by dividing the hermaphroditic adults between two 15 l, 20 °C static seawater baths and gradually raising the temperature of each bath to a maximum of 25 °C. The CO_2 level of each bath was controlled by bubbling the

bath with a microbubbler air stone (RENA, Chalfont, PA, USA) at a rate of 1 l min^{-1} with the appropriate air-CO_2 mixture for 24 h prior to spawning. One bath was bubbled with high CO_2 and the other was bubbled with ambient CO_2. Both baths were covered with clear plastic wrap to help ensure CO_2-equilibration, while allowing observers to view the scallops in the baths. When an individual spawned, it was moved to a 1 l beaker of 20 °C FSW and the spawned gametes were examined to distinguish eggs from sperm. These individual beakers were bubbled with the appropriate (ambient or high) CO_2 level at a rate of 100 ml min^{-1} using a micropipet tip inserted into an airline and were covered with clear plastic wrap to ensure CO_2-equilibration. To prevent self-fertilization, the water in the beakers was frequently changed with pre-CO_2-equilibrated FSW. Eggs were rinsed through a 75 μm filter to collect debris, collected on a 20 μm filter, and subsequently pooled. Seawater with sperm was rinsed through a 20 μm filter to collect debris and the sperm was pooled. Separate sets of filters were used for gametes spawned in ambient and high CO_2 so there was no cross-contamination. For gametes spawned in each CO_2 treatment, sperm and eggs were each pooled separately into about 1 L of seawater, which was bubbled at 100 ml min^{-1} with the appropriate CO_2 level. About 1 ml of sperm spawned in ambient CO_2 was added to the eggs spawned in ambient CO_2 and the embryos were left to develop in the beaker for 45 min, until the embryonic development was at the polar body stage or first cleavage (Belding 1910). The eggs spawned in high CO_2 were simultaneously fertilized with 1 ml of sperm spawned in

high CO_2 and left to develop for about 45 minutes. Each beaker of developing embryos was bubbled with the appropriate CO_2 level during this time.

This induction of spawning was performed two times, in late May 2012 and late June 2012, resulting in two separate experiments, henceforth referred to as Experiment 1 and Experiment 2. For Experiment 1, ambient CO_2-spawned eggs were collected from 2 individuals, although most of the eggs came from 1 of these individuals, and ambient CO_2-spawned sperm was collected from 2 individuals. High CO_2-spawned eggs were collected from 1 individual and high CO_2-spawned sperm was collected from 1 individual. For Experiment 2, ambient CO_2-spawned eggs were collected from 3 individuals and ambient CO_2-spawned sperm was collected from 3 individuals. High CO_2-spawned eggs were collected from 2 individuals and high CO_2-spawned sperm was collected from 5 individuals. Ideally we would want at least 2 adults contributing each type of gamete to each treatment, to identify an overall effect of high CO_2 beyond any maternal effects, so the results of Experiment 1 are interpreted cautiously. For both experiments, if a scallop released both eggs and sperm, only the eggs were used.

3.3.5 Assessment of Fertilization Success and Embryo Density Estimation

When the scallop embryos were 45 min post-fertilization, they were homogeneously suspended in the beaker by the up and down motion of a graduated cylinder (Helm and Bourne 2004) and 1 ml was removed to assess fertilization success and to estimate embryo density. Embryos were counted at 100X

magnification on a gridded slide in which each grid square held 1 µl (Widman et al. 2001). An embryo was considered fertilized by evidence of a polar body or by evidence of first or second cleavage (Belding 1910). The total number of embryos seen and the total number of unfertilized embryos were counted. This count was performed twice per experiment, once for embryos fertilized in ambient CO_2 conditions and once for embryos fertilized in high CO_2 conditions. These counts were used to calculate the percent of embryos from each CO_2 treatment that were successfully fertilized.

3.3.6 Larval Culture

When the embryos were about 2 h old, they were stocked at an initial density of 30 embryos ml^{-1} and were maintained in 800 ml of 0.35 µm FSW in 1-l covered polyethylene cups, which were previously conditioned in running seawater for at least four weeks. Filtered seawater was pre-CO_2 equilibrated by bubbling with the appropriate air-CO_2 mixture in covered 14-l buckets for 24 h prior to filling the 800 ml culture cups. Embryos that had been fertilized in ambient CO_2 were stocked into 5 culture cups containing ambient CO_2 water and 5 cultures cups containing high CO_2 water. Embryos that had been fertilized in high CO_2 were stocked into 5 cultures cups containing ambient CO_2 water and 5 culture cups containing high CO_2 water. This resulted in four experimental treatments: ambient CO_2-fertilized/ambient CO_2-grown (ambient treatment), high CO_2-fertilized/high CO_2-grown (high-CO_2 treatment), ambient CO_2-fertilized/high CO_2-grown (switch-to-

high-CO_2 treatment), and high CO_2-fertilized/ambient CO_2-grown (switch-to-ambient treatment). Each culture cup was bubbled with the appropriate air or CO_2-compressed air mixture for the 7-d duration of the experiment at a rate of approximately 100 ml min^{-1}.

Cultures were fed daily with laboratory-raised *Isochrysis galbana* (Tahitian strain, T-iso) in the exponential phase of growth at a density of 37,500 cell ml^{-1}. This ration has been shown to produce good growth rates and survivorship of bay scallop larvae (Widman et al. 2001). Culture water was changed every two days with pre-CO_2 equilibrated FSW from the 14 l buckets. Prior to water changes, the carbonate chemistry of the pre-equilibrated water in the 14 l buckets was measured as described above. During water changes, each culture was gently poured through a 20 μm sieve, which caught the larvae. The larvae were rinsed back into the cup and the cup was filled to 800 ml. To maintain a stable temperature, all culture cups were contained in a water bath controlled by an aquarium chiller/heater (T = 23.7 ± 0.3 °C).

3.3.7 Microscopic Imaging and Shell Measurements

At 1, 3, and 7 d, approximately 75 larvae from each culture were preserved in 95 % ethanol for microscopic imaging and shell measurement. Imaging and shell measurements were carried out as described in section 2.3.5 of Chapter 2. Preliminary experiments showed that four replicates gave sufficient statistical power to detect differences in mean larval size. A fifth culture cup was included in

each treatment to ensure that if one culture crashed, four would remain available for size measurements. Mean growth rate (μm d^{-1}) for each replicate was calculated as the increase in mean shell length from 1 d to 7 d, divided by the number of days (6 d).

3.3.8 Survival Estimation

Percent survival was estimated at 1, 3, and 7 d according to the method described in section 2.3.6 of Chapter 2. Survival estimates from all five replicate cultures were included in analyses to improve statistical power. Due to an error in quantifying initial stocking densities during Experiment 2, survival estimates are calculated only for Experiment 1.

3.3.9 Statistical Analysis

All statistical analyses were performed using Systat® 13 Software (Systat Software, Inc., Chicago, IL, USA). Percent survival data were arcsine-square root-transformed prior to statistical analyses. Repeated measures ANOVA tests were run to compare survival and shell length (separately) among the four treatments at 1, 3, and 7 d. One-way ANOVAs followed by Tukey's Highly Significant Difference tests were run separately for each date to compare survival and shell length among the four treatments. One-way ANOVAs were run separately for each experiment to compare growth rates from days 1-7 among the four treatments.

3.4 Results

3.4.1 Fertilization Success

The percent of eggs fertilized was reduced for gametes exposed to high CO_2 relative to gametes exposed to ambient CO_2 in both Experiment 1 and Experiment 2 In Experiment 1, the percent of eggs fertilized was 94.5 % and 74.5 % for gametes exposed to ambient CO_2 or high CO_2, respectively. In Experiment 2, the percent of eggs fertilized was 98.2 % and 95.7 % for gametes exposed to ambient CO_2 or high CO_2, respectively. Because these measurements were not replicated within either experiment and because the extent of the difference in fertilization success was not consistent between the two experiments, we interpret these results cautiously.

3.4.2 Shell Development and Length

Development through larval stages (Fig. 3.1) in all treatments progressed typically for this species (Belding 1910; Widman et al. 2001). At 1 d, most larvae were in the fully shelled veliger stage, while those larvae that were still in the trochophore stage (not pictured) showed evidence of calcification, visualized as birefringence under cross-polarized light. By day 3, all larvae were post-D-stage, although some larvae had unequal-sized valves (for example, the 3 d switch-to-high-CO_2 larva in Fig. 3.1A). When larvae exhibited unequal-sized valves, the shell length of the larger valve was measured. Nearly all larvae in the high CO_2 and switch-to-high-CO_2 treatments displayed a dark spot or line near the hinge under transmitted

light (Fig. 3.1A, C) that was also evident under cross-polarized light (Fig. 3.1B, D). Based on subsequent scanning electron microscopy visualization of a subset of larvae from each treatment from Experiment 1, it was determined that this dark spot or line was an indicator of a dorso-ventrally oriented abnormal indentation of the larval shell located near the hinge. Scanning electron micrographs are presented in Fig. 4.3 in Chapter 4. This abnormal indentation was rarely seen on larvae from the ambient and switch-to-ambient treatments, although the frequency of occurrence was not quantified.

The CO_2 conditions experienced during fertilization did not have an impact on larval growth or size (Fig. 3.2, Table 3.2). However, exposure to high CO_2 conditions during subsequent larval development (starting ~2 h post-fertilization) caused a decrease in shell size relative to exposure to ambient CO_2 conditions, regardless of CO_2 exposure during fertilization (Experiment 1: repeated measures ANOVA, Wilk's Lambda = 0.0117, F = 14.187, df = 9, 24, $p < 0.00001$; Experiment 2: repeated measures ANOVA, Wilk's Lambda = 0.0052, F = 20.888, df = 9, 24, $p < 0$ .00001). This decrease in shell size was seen consistently throughout the 7 d duration of both Experiment 1 and Experiment 2. For Experiment 1, the mean shell lengths of larvae from the ambient and switch-to-ambient treatments were significantly larger than the shell lengths of larvae from the switch-to-high CO_2 and high-CO_2 treatments at 1 and 3 d (Fig. 3.2A, Tables 3.2, 3.3). At 7 d, the mean shell lengths of larvae from the ambient treatment were significantly larger than the shell lengths of larvae from the high-CO_2 treatment. For Experiment 3.2, the mean shell

lengths of larvae from the ambient and switch-to-ambient treatments remained significantly larger than the shell lengths of larvae from the switch-to-high-CO_2 and high-CO_2 treatments throughout the entire experiment (Fig. 3.2B, Tables 3.2, 3.3). At 1 and 3 d, larvae from the high-CO_2 treatment were significantly larger than larvae from the switch-to-high-CO_2 treatment; this difference in size was no longer significant by day 7 (Fig. 3.2B). No significant difference was seen in mean shell growth rates (Table 3.2) integrated over days 1-7 (Experiment 1: one-way ANOVA, $F = 0.97$, df = 3, $p = 0.44$; Experiment 2: one-way ANOVA, $F = 1.97$, df = 3, $p = 0.17$).

3.4.3 Larval Survival

Exposure to high CO_2 during fertilization resulted in consistently lower larval survival from day 1-7, relative to exposure to ambient CO_2 during fertilization (Fig. 3.3, Table 3.4). This difference was significant on days 1 and 3, and on day 7, survival in the continuous ambient treatment was significantly higher than survival in all other treatments (Fig. 3.3). Additionally, within each fertilization group (ambient or high CO_2) survival was lower in those treatments where larvae subsequently developed in high CO_2. On day 1, survival was significantly lower in the high-CO_2 treatment, relative to the switch-to-ambient treatment and in the switch-to-high-CO_2 treatment, relative to the ambient treatment. This significant decrease in survival persisted through days 3 and 7 with regard to the switch-to-high-CO_2 and ambient treatments.

3.5 Discussion

In both Experiments 1 and 2, fertilization success was reduced when gametes were exposed to high CO_2 conditions prior to and during fertilization. However, the extent to which fertilization success was reduced was not consistent between experiments. This variable fertilization success could be due to variable gamete quality (Havenhand and Schlegel 2009), but is also commonly a result of variable compatibilities between male and female gametes (Styan et al. 2008). A reduction in fertilization success as a result of exposure to high CO_2 has previously been documented in the clam *M. balthica* (Van Colen et al. 2012) and the oysters *S. glomerata* and *C. gigas* (Parker et al. 2009; Parker et al. 2010). In contrast, other groups have not observed a CO_2 exposure-induced reduction in fertilization success of *C. gigas* (Kurihara et al. 2007; Havenhand and Schlegel 2009), perhaps due to intraspecific differences among oyster populations. Because the fertilization success measurements in the current experiments were not replicated, they cannot be attributed definitively to differences in CO_2 exposure.

Exposure of gametes to high CO_2 conditions prior to and during fertilization did not negatively impact subsequent larval development and growth. Instead, larval development and growth were influenced by CO_2 conditions experienced during larval development (e.g., after 2 h post-fertilization), with larvae in both the switch-to-high-CO_2 and high-CO_2 treatments having significantly smaller shells than larvae in both the ambient and switch-to-ambient treatments (Fig. 3.2). This

significant effect was seen in both Experiment 1 and Experiment 2 for days 1 and 3 and in Experiment 2 on day 7.

Impacts of exposure to high CO_2 during larval development (starting at 2 h post-fertilization) on larval shell size were evident starting when larvae were just 24 h old in both experiments. While the size differences persisted throughout the experiment, growth rates from day 1-7 were not significantly different among any treatments in either experiment, indicating that the size differences at the end of the first week of larval development were a function of differences that appeared within the first day of larval development. Similar to the size results from the 3-d Switch Experiment (Chapter 2), it appears that growth during the first day of larval development, when the larvae are initiating calcification and building their first shell (Belding 1910; Widman et al. 2001), is critical in determining the size that larvae ultimately reach within the first week of development. Calcium carbonate crystals cause cross-polarized light to birefringe (Weiss et al. 2002), which can be used as a qualitative indication of calcification. The shells of 1 d old larvae from the ambient and switch-to-ambient treatments show stronger birefringence than those of 1 d old larvae from the switch-to-high-CO_2 and high-CO_2 treatments (Fig. 3.1B, D). This indicates more calcification of the 1 d old larval shell when larvae were exposed to ambient CO_2.

While high CO_2 exposure during fertilization did not impact larval shell size, it did affect larval survival. In Experiment 1, survival was significantly reduced when gametes were fertilized in high CO_2 (switch-to-ambient and high-CO_2

treatments) compared with survival of gametes that were fertilized under ambient CO_2 (ambient and switch-to-ambient treatments). This difference was seen as early as day 1 and lasted through day 3. On day 7, survival in the ambient treatment was significantly higher than survival in all other treatments. Within a fertilization group (high or ambient CO_2), exposure to high CO_2 during larval development reduced survival of larvae, compared to those exposed to ambient CO_2 during larval development. This suggests that unlike growth, larval survival is influenced by CO_2 exposure during both fertilization *and* larval development.

However, an alternative explanation for the trend seen in larval survival is that the effects are maternal effects as opposed to treatment effects. Because the majority of eggs fertilized in ambient CO_2 came from one scallop, and all of the eggs fertilized in high CO_2 came from one (different) scallop, a difference in gamete quality could have a marked effect on larval survival (Barber and Blake 2006). While we were unable to estimate survival in Experiment 2, we feel that the strong replication of size trends between the two experiments when different parent scallops were used is an indication that the observed effects were treatment effects, not maternal effects.

As previously pointed out in Chapter 2, a decrease in the size of scallop larvae exposed to high CO_2 may have indirect effects on subsequent survival in the field, due to delayed metamorphosis and therefore, increased vulnerability to predation (Thorson 1950; Sastry 1965). Additionally, if the smaller larvae resulted in smaller

adults, reproductive output of those individuals could be stunted simply because the size of the mature gonad is proportional to the size of the adult scallop (Barber and Blake 2006). A factor that could further indirectly impact survival is the abnormal shell curvature seen near the hinge of shells of larvae reared in high CO_2 (Fig. 3.1A, C). The bivalve hinge allows the larval shell to open and close, facilitating the intake of food particles and release of waste (Cragg 2006). An abnormality in the hinge region of the shell could prevent larvae from properly obtaining the food particles necessary for survival.

Our observation of a negative effect of high CO_2 during fertilization on larval shell size is consistent with other studies of bivalves (Fig. 3.4), although the sensitivity of the response varied by species. The magnitude of this CO_2 effect on early larvae of the Pacific oyster (*C. gigas*, Kurihara et al. 2007) was similar to that observed in our experiments. The effect on mussel larvae (*M. galloprovincialis*, Kurihara et al. 2008), however was slightly greater; the early mussel veliger larvae exposed to high CO_2 were smaller relative to ambient conditions than early bay scallop and oyster larvae (Fig. 3.4). The difference in sensitivity among species was relatively small and the same negative size trend with increasing CO_2 was seen in all three of these species, each of which represents a different order of bivalve. This indicates that a decrease in larval shell size could be a consistent response to exposure to high CO_2/low $\Omega_{aragonite}$ during fertilization and early larval development across bivalve orders.

Kurihara et al. (2007) also found that *C. gigas* larvae in the high CO_2 treatment had abnormally formed shells, similar to the abnormally indented *A. irradians* shells described here. If similar processes affect oyster larvae, it is likely that these effects are a result of exposure to high CO_2 during early larval development, not of exposure during fertilization. Interestingly, this shell abnormality was not reported for *M. galloprovincialis* larvae (Kurihara et al. 2008), indicating that mussel shell development may not be affected by early exposure to high CO_2 in the same way that bay scallop and Pacific oyster shell development is affected.

It is important to remember that OA is concurrently taking place with warming temperatures and there is evidence that these stressors may act synergistically. Parker et al. (2010) found that with the oyster *S. glomerata*, exposure to high CO_2 and suboptimal temperature had lethal effects on larval development *only* when the larvae resulted from gametes fertilized in high CO_2 treatments, indicating that the high CO_2 fertilization had a lasting effect on larval development when combined with another stressor.

Bay scallops living in coastal and estuarine environments will face increasingly unfavorable conditions in coming years as atmospheric CO_2-induced OA exacerbates already-high pCO_2 conditions in these locales. While larval development appears to be affected most strongly by CO_2 conditions experienced after fertilization has taken place, larval survival is impacted when gametes are exposed to high CO_2 during fertilization. Larvae that were spawned in regions of

high CO_2 may be at an ecological disadvantage to those spawned in more exposed regions with lower CO_2 conditions. Hatchery managers may want to carefully control the CO_2 conditions in which adult scallops spawn and larvae develop in order to ensure survival and successful development of larvae.

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Table 3.1. Mean (± SD) water chemistry for the ambient and high CO_2 conditions during Experiments 1 and 2. Values for the spawning baths reflect the conditions in which adult scallops were induced to spawn; values for buckets represent the equilibrated water used to replace old culture water at water changes; values for culture cups represent the water in culture cups just prior to each water change; values for blank cups represent the water in culture cups with no larvae or algae added, just prior to each water change. pH, total alkalinity (A_T), temperature, and salinity were measured; all other data were calculated from pH and alkalinity using CO2SYS software (Pierrot et al. 2006) with K_1, K_2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), $KHSO_4$ from Dickson (1990), and pH measured on the seawater scale. ($[HCO_3^-]$ = bicarbonate ion concentration; $[CO_3^{2-}]$ = carbonate ion concentration; $[CO_2]$ = dissolved carbon dioxide concentration; $\Omega_{aragonite}$ = aragonite saturation state.)

Parameter	Ambient Spawning Bath	Ambient Buckets	Ambient Culture Cups	Switch to Ambient Culture Cups	Ambient Blank Cups	High CO_2 Spawning Bath	High CO_2 Buckets	High CO_2 Culture Cups	Switch to High CO_2 Culture Cups	High CO_2 Blank Cups
Experiment 1										
n	1	8	14	15	2	1	8	15	14	3
pH	7.933	7.965 ± 0.006	7.954 ± 0.011	7.954 ± 0.017	7.959 ± 0.026	7.246	7.306 ± 0.027	7.287 ± 0.022	7.281 ± 0.012	7.284 ± 0.008
A_T (µEq kg^{-1})	2111.4	2135.0 ± 16.5	2149.0 ± 21.8	2157.0 ± 17.8	2182.3 ± 25.9	2119.8	2131.4 ± 12.1	2157.6 ± 16.8	2175.1 ± 31.1	2132.6 ± 6.7
Temperature (°C)	19.8	23.6 ± 0.5	23.8 ± 0.3	23.6 ± 0.2	23.8 ± 0.3	20.2	23.5 ± 0.5	23.7 ± 0.3	23.7 ± 0.3	23.7 ± 0.2
Salinity	31.48	31.67 ± 0.27	32.10 ± 0.29	32.06 ± 0.30	32.47 ± 0.50	31.44	31.66 ± 0.23	32.04 ± 0.28	32.33 ± 0.47	31.71 ± 0.21
pCO_2 (µatm)	499.4	462.5 ± 7.4	478.1 ± 12.6	480.1 ± 20.6	478.5 ± 29.1	2774.7	2457.1 ± 156.5	2597.9 ± 129.7	2653.9 ± 58.4	2591.6 ± 49.9
DIC (µmol kg^{-1})	1937.9	1919.6 ± 13.4	1933.8 ± 15.2	1942.6 ± 11.2	1963.1 ± 17.0	2165.7	2146.8 ± 13.3	2177.8 ± 17.6	2196.7 ± 28.3	2154.0 ± 4.7
$[HCO_3^-]$ (µmol kg^{-1})	1792.3	1749.0 ± 12.4	1762.8 ± 11.5	1771.7 ± 10.0	1787.2 ± 5.2	2044.1	2033.3 ± 11.7	2061.2 ± 16.2	2078.6 ± 27.3	2038.6 ± 4.9
$[CO_3^{2-}]$ (µmol kg^{-1})	129.1	156.8 ± 4.5	156.9 ± 5.3	156.6 ± 6.7	161.7 ± 12.6	30.7	39.9 ± 3.0	39.3 ± 2.0	39.4 ± 1.9	38.2 ± 0.8
$[CO_2]$ (µmol kg^{-1})	16.5	13.8 ± 0.3	14.2 ± 0.4	14.3 ± 0.6	14.2 ± 0.8	90.9	73.6 ± 5.2	77.3 ± 3.9	78.8 ± 1.9	77.1 ± 1.3
$\Omega_{aragonite}$	2.04	2.52 ± 0.08	2.52 ± 0.08	2.51 ± 0.10	2.59 ± 0.19	0.49	0.64 ± 0.05	0.63 ± 0.03	0.63 ± 0.03	0.61 ± 0.02
Experiment 2										
n	1	5	15	15	3	1	5	15	15	3
pH	7.925	7.953 ± 0.014	7.974 ± 0.009	7.965 ± 0.010	7.972 ± 0.009	7.251	7.308 ± 0.005	7.290 ± 0.009	7.295 ± 0.015	7.286 ± 0.006
A_T (µEq kg^{-1})	2114.4	2133.1 ± 12.0	2214.5 ± 35.7	2184.6 ± 29.5	2184.5 ± 19.2	2127.2	2122.3 ± 13.9	2184.6 ± 23.1	2179.3 ± 34.4	2145.3 ± 10.9
Temperature (°C)	21.4	25.1 ± 0.1	24.3 ± 0.1	24.3 ± 0.1	24.3 ± 0.1	21.6	25.1 ± 0.0	24.3 ± 0.1	24.3 ± 0.1	24.3 ± 0.1
Salinity	31.87	32.04 ± 0.19	33.24 ± 0.51	32.89 ± 0.47	32.72 ± 0.22	31.84	31.90 ± 0.11	32.62 ± 0.23	32.69 ± 0.46	32.24 ± 0.14
pCO_2 (µatm)	509.1	475.8 ± 19.0	462.3 ± 8.0	468.9 ± 10.8	459.6 ± 7.7	2764.9	2443.4 ± 29.2	2598.0 ± 37.6	2568.5 ± 89.7	2590.3 ± 36.7
DIC (µmol kg^{-1})	1931.8	1910.5 ± 12.9	1973.9 ± 26.2	1952.8 ± 21.8	1949.9 ± 12.4	2165.7	2130.7 ± 13.4	2190.1 ± 20.6	2193.1 ± 33.2	2162.6 ± 10.7
$[HCO_3^-]$ (µmol kg^{-1})	1780.7	1735.5 ± 15.0	1786.2 ± 19.8	1771.2 ± 17.2	1766.7 ± 8.0	2045.7	2018.2 ± 12.8	2073.1 ± 20.0	2076.3 ± 31.4	2046.8 ± 10.0
$[CO_3^{2-}]$ (µmol kg^{-1})	135	161.4 ± 5.1	174.2 ± 7.2	168.0 ± 6.5	169.9 ± 4.8	33.1	42.4 ± 0.8	41.3 ± 1.4	42.0 ± 1.9	40.1 ± 0.8
$[CO_2]$ (µmol kg^{-1})	16.1	13.7 ± 0.6	13.4 ± 0.3	13.6 ± 0.3	13.4 ± 0.3	86.9	70.1 ± 0.9	75.7 ± 1.1	74.8 ± 2.5	75.7 ± 1.2
$\Omega_{aragonite}$	2.15	2.61 ± 0.08	2.79 ± 0.11	2.69 ± 0.10	2.72 ± 0.07	0.53	0.68 ± 0.01	0.66 ± 0.02	0.67 ± 0.03	0.64 ± 0.02

Table 3.2. Mean (± SD) size (µm) at 1, 3, and 7 d and mean (± SD) growth rate (µm d^{-1}) from 1-7 d of *Argopecten irradians* larvae raised in four CO_2 treatment regimes; *n* = 4 replicate culture containers with measurements made on at least 15 larvae from each.

Treatment	Day 1	Day 3	Day 7	Growth Rate
Experiment 1				
Ambient	91.8 ± 2.7	105.8 ± 4.0	136.7 ± 7.1	7.5 ± 1.6
Switch to High CO_2	70.2 ± 1.9	89.2 ± 1.8	123.0 ± 6.6	8.8 ± 1.3
Switch to Ambient	87.4 ± 1.3	102.2 ± 0.5	132.3 ± 7.8	7.5 ± 1.4
High CO_2	67.9 ± 3.8	83.0 ± 4.5	119.7 ± 5.2	8.6 ± 1.4
Experiment 2				
Ambient	87.5 ± 2.1	101.3 ± 1.3	132.3 ± 1.1	7.5 ± 0.2
Switch to High CO_2	65.7 ± 2.3	76.0 ± 2.8	104.3 ± 7.5	6.4 ± 1.4
Switch to Ambient	87.9 ± 1.6	103.7 ± 4.1	123.8 ± 4.0	6.0 ± 0.9
High CO_2	73.5 ± 1.1	86.5 ± 2.0	112.5 ± 3.1	6.5 ± 0.5

Table 3.3. One-way ANOVAs of mean shell length (µm) of *Argopecten irradians* larvae from Experiments 1 and 2 raised in four CO_2 treatment regimes (Ambient/Ambient CO_2, Ambient/High CO_2, High/Ambient CO_2, and High/High CO_2) at 1, 3, and 7 d; n = 4.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
Experiment 1						
Day 1	Treatment	1734.69	3	578.23	85.97	<0.001
	Error	80.71	12	6.73		
Day 3	Treatment	1392.15	3	464.05	46.69	<0.001
	Error	119.27	12	9.94		
Day 7	Treatment	752.42	3	250.81	5.49	0.013
	Error	548.23	12	45.69		
Experiment 2						
Day 1	Treatment	1437.10	3	479.03	143.06	<0.001
	Error	40.18	12	3.35		
Day 3	Treatment	2035.41	3	678.47	88.87	<0.001
	Error	91.61	12	7.63		
Day 7	Treatment	1822.33	3	607.44	29.13	<0.001
	Error	250.25	12	20.85		

Table 3.4. One-way ANOVAs of mean percent survival (arcsine-square root-transformed) of *Argopecten irradians* larvae from Experiment 1 raised in four CO_2 treatment regimes (Ambient/Ambient CO_2, Ambient/High CO_2, High/Ambient CO_2, and High/High CO_2) at 1, 3, and 7 d; n = 5.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
Day 1	Treatment	0.84	3	0.28	122.78	<0.001
	Error	0.04	16	0.00		
Day 3	Treatment	1.04	3	0.35	73.31	<0.001
	Error	0.08	16	0.00		
Day 7	Treatment	0.50	3	0.17	33.74	<0.001
	Error	0.08	16	0.00		

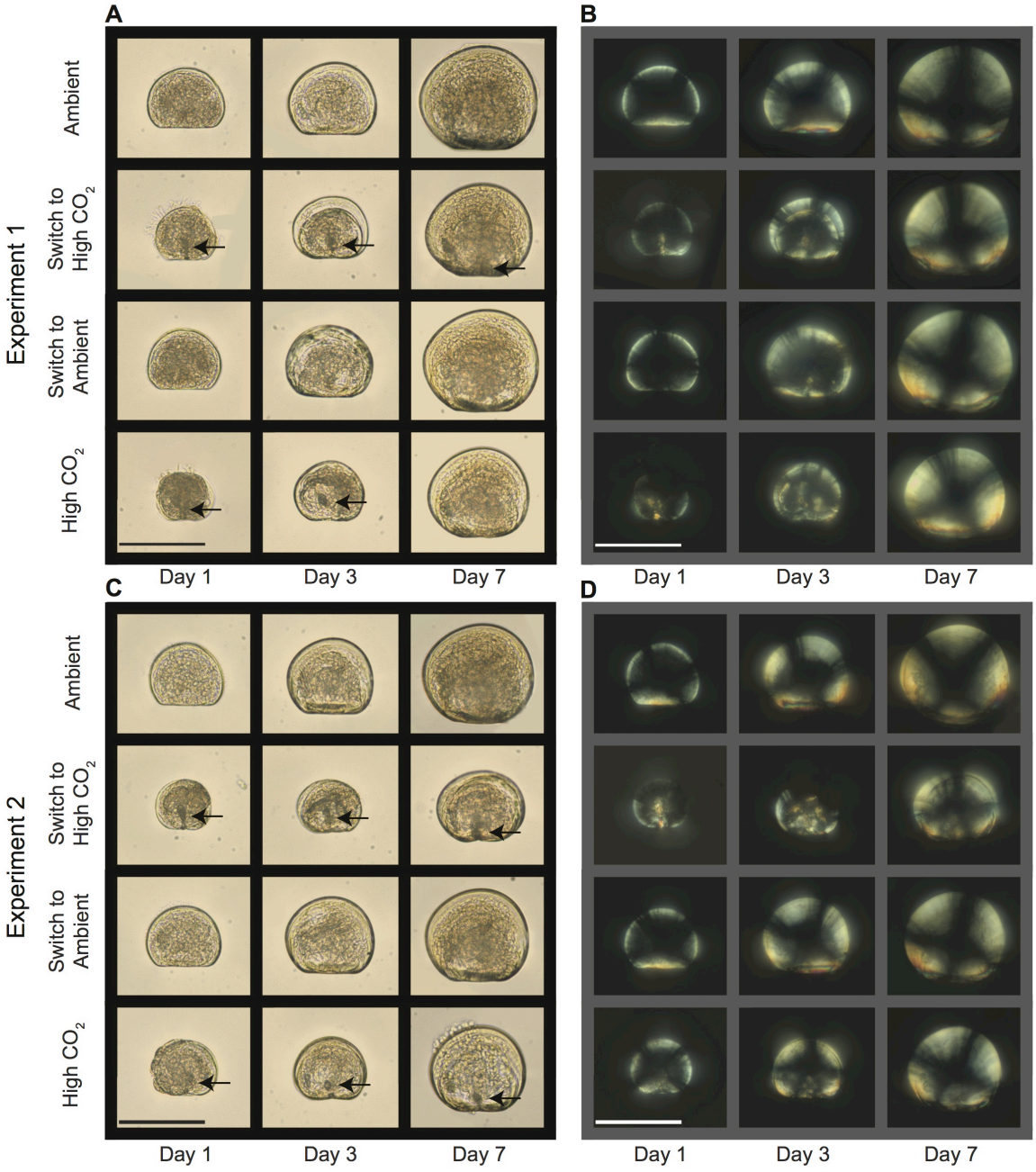

Figure 3.1. Larval morphology of bay scallops (*Argopecten irradians*) exposed to varied pCO_2 conditions from Experiment 1 (A-B) and Experiment 2 (C-D), viewed under both transmitted light (A, C) and cross-polarized light (B, D). Larvae were preserved in 95 % ethanol after incubation for 1, 3, or 7 d in one of the four CO_2 treatment regimes (ambient, switch-to-high-CO_2, switch-to-ambient, and high-CO_2). Larvae shown represent the mean shell length for each treatment and age. Arrows indicate the dark spot on the larval shell that represents a shell abnormality. Images are all to the same scale; scale bar = 100 µm.

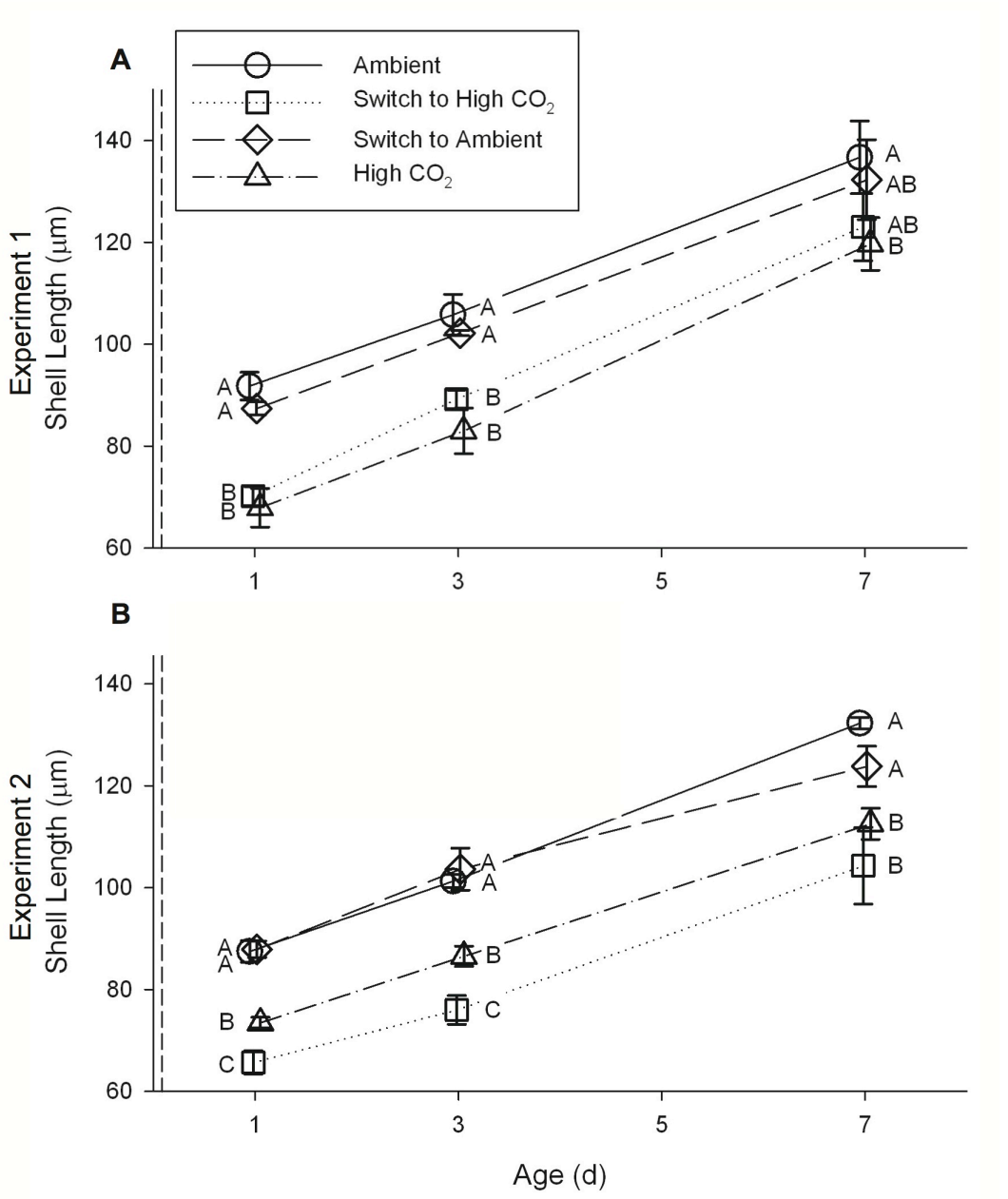

Figure 3.2. Shell length of larval bay scallops (*Argopecten irradians*) from Experiment 1 (A) and Experiment 2 (B) during the first week of larval development. Values are mean ± SD of 4 replicate culture containers. The dashed vertical line at t = 2 h indicates the age at the time of inoculation into culture cups, which was also the age at which CO_2 conditions were switched for the two switch treatments. Different letters (A, B, C) denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 3), followed by Tukey's HSD test.

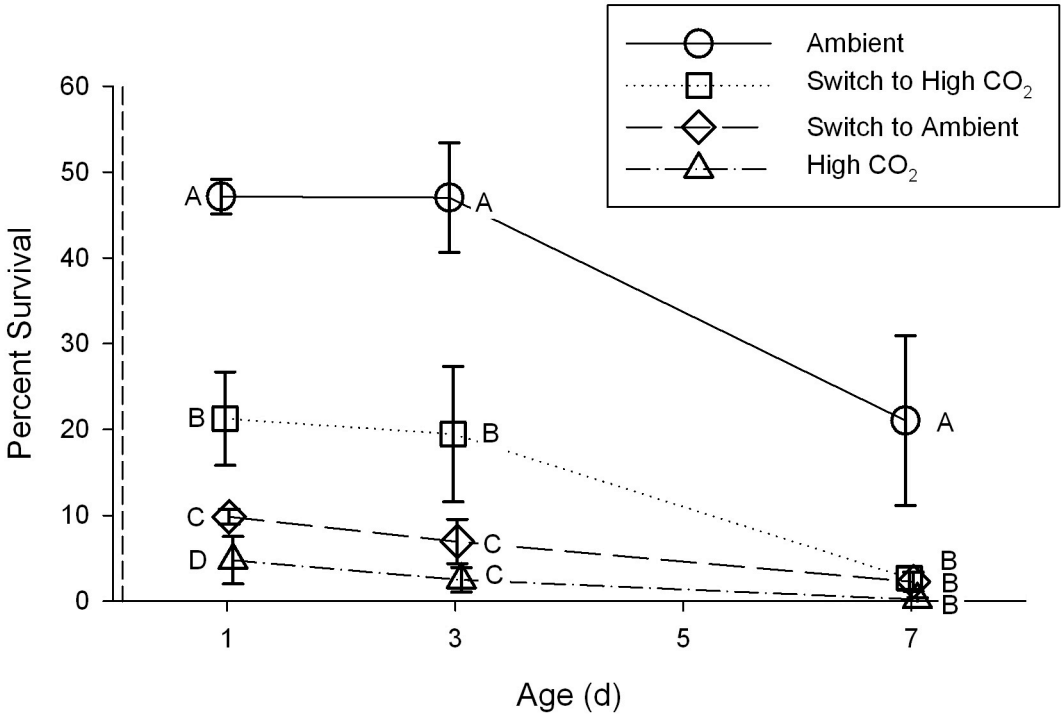

Figure 3.3. Survival of larval bay scallops (*Argopecten irradians*) from Experiment 1, expressed as the percent of larvae surviving from the time of inoculation (age = 2 h), during the first week of larval development. Values are mean ± SD of $n = 5$ replicate culture containers. The dashed vertical line at t = 2 h indicates the age at the time of inoculation into culture cups, which was also the age at which CO_2 conditions were switched for the two switch treatments. Letters denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 4), followed by Tukey's HSD test.

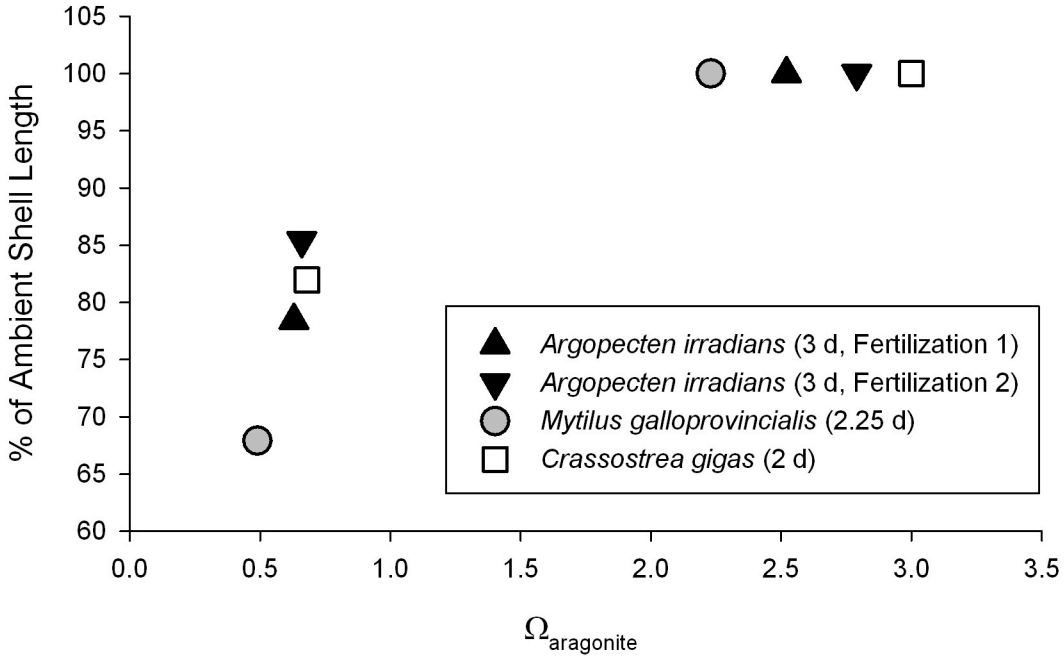

Figure 3.4. Shell length of early veliger larvae of three bivalve species, expressed as % of ambient shell length, where 'ambient' refers to the experimental treatment with water pCO_2 closest to the current atmospheric pCO_2 value. Data for bay scallops (*Argopecten irradians*) are calculated from those data presented in this thesis chapter; data for the mussel *Mytilus galloprovincialis* are calculated from Kurihara et al. (2008); data for the oyster *Crassostrea gigas* are calculated from Kurihara et al. (2007). In all cases, gametes were exposed to treatment conditions (ambient or low $\Omega_{aragonite}$) during fertilization and embryos and larvae were maintained in their respective $\Omega_{aragonite}$ conditions for the duration of the experiment with no switch in conditions (i.e., only the continuous ambient and high-CO_2 treatments from the bay scallop fertilization experiments described in this thesis chapter are shown).

Chapter 4

Isolating critical high pCO_2 exposure windows for larval bay scallops (*Argopecten irradians*)

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In preparation

4.1 Abstract

Ocean acidification, characterized by elevated pCO_2 has variable effects on marine invertebrates. Coastal and estuarine environments, where many commercially and economically important organisms live, experience larger variability and rapid shifts of pCO_2 levels than the open ocean. Increases in pCO_2 have been shown to have negative effects on growth and development in the early life stages of calcifying marine invertebrates such as bivalves, but it is not clear which developmental stage might be most sensitive. We investigate the idea that initial calcification is a critical stage during which exposure to high pCO_2 will have severe and persistent effects on larval growth and development of the bay scallop *Argopecten irradians*. In a set of experiments varying the timing of exposure of embryonic and larval bay scallops to high CO_2 (resulting in pH = 7.3, Ω_{ar} = .65), exposure to high CO_2 during initial calcification (12-24 h post-fertilization) results in significantly smaller shells, relative to ambient conditions (pH = 7.96, Ω_{ar} = 2.60), and this decrease in size persists through the first week of larval development. However, exposure to high CO_2 at 2-12 h post-fertilization (prior to calcification) does not impact shell size. This suggests that the CO_2 impact on size is a direct consequence of water chemistry during calcification, not a consequence of earlier exposure. Initial exposure to high CO_2 (2-12 h post-fertilization) causes an abnormal indentation in shells of some larvae, even if they are exposed to ambient conditions during initial calcification. This impact does not occur in response to CO_2 exposure after the 2-12 h period of pre-calcification development.

4.2 Introduction

Coastal and estuarine systems, and the organisms living therein, are subject to fluctuating physical and chemical conditions, including aqueous carbonate chemistry (Cai and Wang 1998; Howarth et al. 2011; Hofmann et al. 2011). Carbonate chemistry fluctuates as a result of both natural and anthropogenic processes on timescales ranging from daily to seasonal (Melzner et al. 2012). For instance, phytoplankton photosynthesize during daylight hours, drawing down CO_2 and releasing O_2, while during the night, autotrophs and heterotrophs respire, drawing down O_2 and releasing CO_2. As a result of this CO_2 uptake and release, pH typically rises during the day and falls at night. On seasonal scales, increased temperature during summer months leads to increased microbial respiration of organic matter, and seasonal stratification can produce sub-surface regions of O_2 depletion, CO_2 elevation, and pH drop (Dai et al. 2006; Diaz and Rosenberg 2008; Howarth et al. 2011; Melzner et al. 2012). Coastal and estuarine environments are further affected by a reduction in pH caused by increased atmospheric CO_2 levels resulting from fossil fuel burning (Caldeira and Wickett 2003; Doney et al. 2009; Feely et al. 2009). Ocean acidification (OA), affects coastal and open oceans alike, but coastal regions may be more vulnerable due to their relatively low buffering capacity (Zeebe and Wolf-Gladrow 2005; Borges and Gypens 2010; Cai et al. 2011; Melzner et al. 2012; Sunda and Cai 2012)

relative to the open ocean. Therefore, OA is likely to decrease pH levels of coastal and estuarine systems to a greater extent than for open ocean systems (Cai et al. 2011; Melzner et al. 2012).

An important consequence of increased CO_2 in seawater is a decrease in the calcium carbonate saturation state (Ω) (Doney et al. 2009). This drop in Ω can make calcification (shell-building) more difficult, or more energetically costly, for marine organisms (Orr et al. 2005; Gazeau et al. 2007; Waldbusser et al. 2011). Also, many calcifying marine invertebrates, including bivalves, spawn during summer months (Belding 1910; Costello and Henley 1971) when pCO_2 levels are at the highest and Ω is the lowest in temperate systems (Feely et al. 2010; Waldbusser et al. 2011; Melzner et al. 2012).

Recent evidence suggests that early life stages of marine invertebrates, particularly of bivalve molluscs, which produce calcareous shells, are negatively affected by high CO_2 conditions. Negative effects of OA have been shown on all early life stages of bivalves, including fertilization, D-stage (early) development, later larval development, and juvenile development (Parker et al. 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al. 2010; Parker et al. 2010; Waldbusser et al. 2011; Gazeau et al. 2011; Van Colen et al. 2012), although some bivalve species appear to be more tolerant to OA conditions than others (Miller et al. 2009). The bay scallop (*Argopecten irradians*) larvae experienced delayed metamorphosis when exposed to pH 8.04 relative pH 7.80 (Talmage and Gobler 2010). Exposure to low pH water decreases survival of some bivalve larvae; for instance, survival of the Sydney rock

oyster (*Saccostrea glomerata*) exposed to water with pH 7.6 was reduced by 72 % relative to larvae exposed to water with pH 8.1 (Watson et al. 2009). Growth effects are also apparent in larvae of the clam *Macoma balthica*, which grew more slowly when exposed to water with pH 7.8 or 7.5, relative to pH 8.1 (control) from age 3 d to 19 d (Van Colen et al. 2012). Most studies have exposed larvae to treatments only once they were 1-3 d old, but exposure to low pH during the first day of development, when many bivalve larvae experience the onset of calcification, has an important impact on larval growth.

It is clear that larval bivalves are strongly impacted by high CO_2 conditions, but it is not well understood how variable exposure to high CO_2 conditions affects larval growth, development, and survival, nor at what developmental stage is high CO_2 exposure most critical. Such questions have relevance to both natural ecology and practical applications. In the field, larvae are exposed to variable CO_2 conditions spatially as they are transported through estuaries and temporally as a result of daily fluctuations in photosynthesis and respiration. Many bivalve species are good candidates for aquaculture, often reared as larvae in commercial hatcheries (Shumway and Parsons 2006). Hatcheries would benefit from an understanding of which developmental stages are most vulnerable to high CO_2 conditions and how exposure at different stages may affect larval development.

Here, we address the impact of exposure to variable CO_2 conditions during early larval development on the survival and growth of bay scallop larvae, and identify the stages of early development that are most sensitive to exposure to high

CO_2. The bay scallop is an ideal model organism for this study because of their economic importance as a commercially harvested shellfish. Bay scallops embryos become trochophore larvae when they are about 12 h old and calcification initiates soon thereafter (Belding 1910; Waller 1976; Bellolio et al. 1993). By the time larvae are 24 h old, the shell is typically fully formed and they are considered early D-stage veligers (Belding 1910; Waller 1976; Bellolio et al. 1993). Previous work (Chapter 2 of this thesis) showed that 3-d exposure to high CO_2 caused bay scallop larvae to have significantly smaller shells than those exposed to ambient CO_2 and this size difference persisted after larvae were transferred to ambient CO_2 conditions. Perhaps more importantly, the difference in shell size was seen by the time larvae were 24 h old, which could indicate that the initial calcification period (12-24 h post-fertilization) is particularly sensitive to high CO_2 exposure.

The results of the two experiments described in this thesis chapter show that exposure to high CO_2 during initial calcification (12-24 h post-fertilization) significantly reduces bay scallop larval shell size and that this size difference persists throughout the first week of development. We also show that exposure of larval bay scallops to high CO_2 prior to shell formation (2-12 h post-fertilization) causes an increase in the occurance of abnormally developed larval shells. Combined, these results isolate two distinct developmental stages in which exposure to high CO_2 causes different effects on bay scallop larvae.

4.3 Methods

4.3.1 Adult Collection and Spawning

Adult *A. irradians* individuals were collected during winter and spring months from coastal waters around Martha's Vineyard and Woods Hole, Massachusetts and were held in submerged cages in Little River, an estuarine river near Waquoit Bay, Massachusetts until needed. Scallops were collected under a research collection permit issued by the Commonwealth of Massachusetts Department of Fish and Game, Division of Marine Fisheries. Several days prior to spawning, the adults were brought to Woods Hole Oceanographic Institution, where they were maintained in 16 °C flowing seawater and fed daily with Instant Algae Shellfish Diet (Reed Mariculture, Campbell, CA, USA).

Spawning was induced following the method described in section 2.3.1 of Chapter 2. This spawning procedure was performed for two separate experiments, which will be referred to as the 24-h Switch Experiment and the 12-h Switch Experiment (described in more detail below). For the 24-h Switch Experiment, eggs were collected from 3 individuals and sperm was collected from 4 individuals. For the 12-h Switch Experiment, eggs were collected 5 individuals and sperm was collected from a 5 individuals. In both experiments, if a scallop released both eggs and sperm, only the eggs were used.

When the scallop embryos were 45 min post-fertilization, they were suspended in the beaker by the up and down motion of a graduated cylinder (Helm and Bourne 2004) and 1 ml was removed to assess fertilization success and to

estimate embryo density. Embryos were counted at 100X magnification on a gridded slide in which each grid square held 1 µl (Widman et al. 2001). An embryo was considered fertilized by evidence of a polar body or first or second cleavage (Belding 1910). The total number of embryos seen and the total number of unfertilized embryos were counted. This count was performed once for the 24-h Switch Experiment and twice for the 12-h Switch Experiment. The counts were used to calculate the density of the embryo suspension in order to calculate the volume with which to inoculate each replicate culture container.

4.3.2 Larval Culture

When the embryos were about 2 h old, they were stocked at an initial density of 30 embryos ml^{-1} and were maintained in 800 ml of FSW in 1-l covered polyethylene cups, which had been previously conditioned in running seawater for at least four weeks. Filtered seawater was CO_2-equilibrated by bubbling with the appropriate air-CO_2 mixture in covered 14-l buckets for 24 h prior to filling the culture cups. Each experiment consisted of four experimental treatments: continuous exposure to ambient CO_2 (ambient treatment), continuous exposure to high CO_2 (high-CO_2 treatment), initial exposure to ambient CO_2 followed by exposure to high CO_2 (switch-to-high-CO_2 treatment), and initial exposure to high CO_2 followed by exposure to ambient CO_2 (switch-to-ambient treatment). The two experiments were differentiated by the timing, at 12 or 24 hr, of the change in CO_2 conditions in the switch treatments. Scallop larvae begin to calcify between 12 and

24 hours (Belding 1910; Waller 1976; Bellolio et al. 1993; Widman et al. 2001), so in the 24-h Switch Experiment, larvae initiated calcification prior to the switch, whereas in the 12-h Switch Experiment, the switch occurred prior to the onset of calcification. Each culture cup was bubbled at a rate of approximately 100 ml min^{-1}.

Cultures were fed daily with laboratory-raised *Isochrysis galbana* (Tahitian strain, T-iso) in the exponential phase of growth at a density of 37,500 cell ml^{-1}. This ration has been shown to produce good growth rates and survivorship of bay scallop larvae (Widman et al. 2001). Culture water was changed every two days with CO_2-equilibrated FSW from the 14 l buckets. Prior to water changes, the carbonate chemistry of the pre-equilibrated water in the 14-l buckets and of each replicate culture cup was measured as described below. During water changes, each culture was gently poured through a 20 μm sieve, which caught the larvae. The larvae were rinsed back into the cup and the cup was filled to 800 ml. To maintain a stable temperature, all culture cups were contained in a water bath controlled by an aquarium chiller/heater (T = 23.7 ± 0.5 and 24.2 ± 0.2 °C for the 24-h Switch and 12-h Switch Experiments, respectively). In addition to five replicate culture cups per treatment, 2 cups, one for each CO_2 level, were maintained without larvae or algae added to them to serve as abiological references for the chemistry characterization. The FSW in these cups was analyzed for carbonate chemistry and changed every 2 d, just as the replicate culture cups were.

4.3.3 Manipulation of Water Chemistry

Water chemistry was manipulated as described in section 2.3.3 of Chapter 2. For the high CO_2 exposure, we used 2200 ppm CO_2 , resulting in pH = 7.28 and a calcium carbonate saturation state that was undersaturated with respect to aragonite (Table 4.1). Such pCO_2 values and associated saturation states have been observed in summer months in a local estuary (Childs River, Falmouth, MA, USA) where bivalve larvae are found (McCorkle et al. 2012).

4.3.4 Characterization of Water Chemistry

To characterize the carbonate chemistry of the treatment water, pH, total alkalinity, salinity, and temperature were measured following the procedures described in section 2.3.4 of Chapter 2. Briefly, pH was measured spectrophotometrically following the procedure described by Clayton and Byrne (1993) and Dickson et al. (2007), and using the refit equation of Liu et al. (2011). Alkalinity was measured by Gran titration with 0.01 M HCl. Based on the measured values of pH (seawater scale), total alkalinity, temperature, and salinity, we used CO2SYS Software (Pierrot et al. 2006) to calculate pCO_2, $\Omega_{aragonite}$, and total DIC using the first and second dissociation constants (K_1 and K_2) of carbonic acid in seawater from Mehrbach et al. (1973), refit by Dickson and Millero (1987).

In addition to the replicate culture cups with larvae, 2 cups, one for each CO_2 level, were maintained without larvae or algae added to them to serve as abiotic references for the chemistry characterization. The FSW in these cups was analyzed for carbonate chemistry and changed every 2 d, as for the replicate culture cups.

4.3.5 Light Microscopic Imaging and Shell Measurements

At 1, 3, and 7 d, approximately 50-75 larvae from each culture were preserved in 95 % ethanol for microscopic imaging and shell measurement. Imaging and shell measurements were carried out as described in section 2.3.5 of Chapter 2. Preliminary experiments showed that four replicates gave sufficient statistical power to detect differences in mean larval size. A fifth culture cup was included for each treatment to ensure that if one culture crashed, four would remain available for size measurements. Mean growth rate (μm d^{-1}) for each replicate was calculated as the increase in mean shell length from 1 d to 7 d, divided by the number of days (6 d).

4.3.6 Scanning Electron Microscopy

Upon visualization of larvae through transmitted and polarized light microscopy, a shell abnormality near the hinge was frequently observed in larvae in the high CO_2 and switch to ambient treatments. The frequency of occurrence of this shell abnormality was quantified for all four treatments of each experiment, based on the light microscopy images of ~15 larvae taken from four replicate cultures. However, visualization of larvae through light microscopy was insufficient to identify the nature of the shell abnormality, so a subset of larvae from one replicate of each treatment from the 24-h Switch Experiment were mounted on stubs for scanning electron microscopy (SEM). Approximately 12 larvae from each treatment

(1, 3, and 7 d) were mounted on stubs using double-sided carbon tape and subsequently coated with 5 nm gold using a Leica EM MED020 vacuum coating system. Digital SEM images of the larvave were obtained with a Zeiss NTS Supra40VP electron microscope at 500X magnification using a voltage of 12 kV and an HKL Premium electron backscatter diffraction (EBSD) system.

4.3.7 Survival Estimation

Percent survival was estimated at 1, 3, and 7 d according to the method described in section 2.3.6 of Chapter 2. Survival estimates from all five replicate cultures were included in analyses to improve statistical power.

4.3.8 Statistical Analysis

All statistical analyses were performed using Systat® 13 Software (Systat Software, Inc., Chicago, IL, USA). Percent survival data and percent abnormal shell data were arcsine-square root-transformed prior to statistical analyses. Repeated measures ANOVA tests were run to compare survival and shell length among the four treatments at 1, 3, and 7 d. One-way ANOVAs followed by Tukey's Highly Significant Difference tests were run separately for each date to compare survival and shell length among the four treatments. One-way ANOVAs were run separately for each experiment to compare growth rate among the four treatments.

4.4 Results

4.4.1 Shell Size and Growth

Progression through larval stages (Fig. 4.1) in all treatments was typical for this species (Belding 1910; Widman et al. 2001) in both the 24-h and 12-h Switch Experiments. In the 24-h Switch Experiment, most larvae were in the fully shelled veliger stage at 1 d, while those larvae that were still in the trochophore stage (not pictured) showed evidence of calcification, visualized as birefringence under cross-polarized light. In the 12-h Switch Experiment, all larvae were fully shelled veligers at 1 d. In both experiments, by day 3, all larvae were post-D-stage.

Exposure to high CO_2 conditions during the initial calcification (12-24 h post fertilization) caused a significant decrease in shell size relative to exposure to ambient CO_2 conditions by the time the larvae were 1 d old in both the 12-h and 24-h Switch Experiments (i.e., size in high-CO_2 < size in ambient treatments; Fig. 4.2, Tables 4.2, 4.3). This difference in size persisted throughout the first week of larval development in both experiments (24-h Switch Experiment: repeated measures ANOVA, Wilk's Lambda = 0.056, F = 6.141, df = 9, 24, p < 0.001; 12-h Switch Experiment: repeated measures ANOVA, Wilk's Lambda = 0.005, F = 20.362, df = 9, 24, p < 0.001).

In the 24-h Switch Experiment, at 1 and 3 d, the larvae that were initially exposed to high CO_2 through the onset of calcification (both the switch-to-ambient and high-CO_2 treatments) were not significantly different in size from each other, but were significantly smaller than larvae that were initially exposed to ambient CO_2

(both the ambient and switch-to-high-CO_2 treatments, Fig. 4.2A). By day 7, this pattern remained, but was no longer significant; only the larvae in the switch-to-high-CO_2 treatment were significantly larger than those in the switch-to-ambient treatment. In the 12-h Switch Experiment, it was not the initial CO_2 exposure (2-12 h) that impacted shell size, but rather the CO_2 exposure during the onset of calcification (12-24 h), which occurred after the switch in CO_2 conditions had been carried out (Fig. 4.2B, Tables 4.2, 4.3). Throughout the 7 d duration of the experiment, the shells of larvae exposed to ambient CO_2 during initial calcification (the ambient and switch-to-ambient treatments) were significantly larger than the shells of larvae exposed to high CO_2 during initial calcification (the high-CO_2 and switch-to-high-CO_2 treatments). There was no significant difference in mean growth rates from 1-7 d (Table 4.2) among the four treatments in either the 24-h Switch Experiment or the 12-h Switch Experiment (24-h: one-way ANOVA, F = 2.500, df = 3, p = 0.109; 12-h: one-way ANOVA, F = 1.517, df = 3, p = 0.260).

4.4.2 Shell Development and Structure

Transmitted and cross-polarized light images of larvae in the switch-to-ambient and the high-CO_2 treatments of both Experiments showed an abnormality in the shell development near the hinge at 1, 3, and 7 d (Fig. 4.1). This abnormality appeared in transmitted light images as a dark spot or line near the hinge of the larvae (Fig. 4.1A, C) and in cross-polarized light as a bright spot or line near the hinge of the larvae (Fig. 4.1B, D). SEM visualization of a subset of larvae from the

24-h Switch Experiment (Fig. 4.3) showed this to be an dorso-ventrally oriented, abnormal indentation of the shell near the hinge (Fig 4.3H-L).

Pre-calcification exposure to high CO_2 (2-12 h post-fertilization, i.e., the switch-to-ambient and high-CO_2 treatments) caused a significantly larger percentage of larvae to show this abnormality relative to larvae with initial (2-12 h) exposure to ambient CO_2 in both experiments (Fig. 4.4, Table 4.4). This trend was significant throughout the 7 d duration of both experiments (24-h: repeated measures ANOVA, Wilk's Lambda = 0.016, F = 11.981, df = 9, 24, $p < 0.001$; 12-h: repeated measures ANOVA, Wilk's Lambda = 0.012, F = 13.717, df = 9, 24, $p < 0.001$). The abnormal shell indentation was produced by pre-calcification exposure to high CO_2 in contrast to the decrease in shell size, which was produced by high CO_2 exposure during initial calcification.

SEM visualization of larvae from the 24-h Switch Experiment also showed dissolution of the prodissoconch I of 100 % and 50 % of 7 d larvae imaged from the switch-to-high-CO_2 and high-CO_2 treatments, respectively, (Fig. 4.3F, L). No evidence of dissolution was seen on larvae in either the ambient or switch-to-ambient treatments, nor on the prodissoconch II of any larvae. Shells were visualized from only one replicate of each treatment, so no statistical analyses were performed on prevalence of shell dissolution.

4.4.3 Larval Survival

In both experiments, larvae continuously exposed to ambient CO_2 (the ambient treatment) consistently had higher survival than larvae in all the other treatments (Fig. 4.5). Overall mean survival varied significantly among treatments in both experiments (24-h, repeated measures ANOVA, Wilk's Lambda = 0.212, F = 3.395, df = 9, 34, p = 0.004; 12-h, repeated measures ANOVA, Wilk's Lambda = 0.256, F = 2.853, df = 3, 34, p = 0.013). In the 24-h Switch Experiment, survival was highly variable among replicates within treatments at 1 and 3 d, resulting in no significant difference among treatments on those days. On day 7, survival was low in all replicates, but was significantly higher in the ambient treatment than in the switch-to-ambient and the high-CO_2 treatments (Fig. 4.5A). In the 12-h Switch Experiment, survival was significantly different among treatments at 1, 3, and 7 d (Fig. 4.5B), and was consistently higher in the treatments initially exposed to ambient CO_2 than in the treatments initially exposed to high CO_2.

4.5 Discussion

Exposure to high CO_2 during initial calcification (12-24 h post fertilization) caused a significant decrease in shell size relative to ambient CO_2 exposure. This effect became apparent when the larvae were just 1 d old. Larvae that were held continuously in high CO_2 and those switched into high CO_2 prior to calcification both showed a decrease in size. Larvae that had been exposed to high CO_2, but returned to ambient conditions before calcification, did not show a decrease in shell size. The decrease in shell size persisted throughout the first week of development in both

experiments. However, growth rates from days 1-7 were not different among treatments in either experiment, indicating growth rate was only affected by exposure to high CO_2 during the first day of development. The size difference seen on day 1 persisted throughout the first week simply because larvae in all treatments grew at the same rate after the first day. This demonstration of a significant and persistent CO_2 effect on shell size within the first day of larval development suggests that other studies on bivalve larval development in which CO_2 exposure was initiated only after initial calcification may have underestimated the magnitude of the effects of high CO_2 throughout larval development (Talmage and Gobler 2009; Miller et al. 2009; Watson et al. 2009; Gazeau et al. 2010; Talmage and Gobler 2010; Van Colen et al. 2012).

The size results from the 12- and 24-h Switch Experiments are complementary with the size results from the two Fertilization Experiments and the 3-d Switch Experiment. Together, the data from all four experiments show that it is exposure to high CO_2 during the onset of calcification that determines the larval shell size (Fig. 4.6). When the switch in conditions occurred prior to the onset of calcification (the 12-h Switch and Fertilization Experiments), shell size reflected the final CO_2 exposure conditions; when the switch in conditions occurred after the onset of calcification (the 24-h and 3-d Switch Experiments), shell size reflected the initial CO_2 exposure conditions.

There are several possible explanations for why bay scallop larvae appear to be particularly sensitive to high CO_2 exposure during initial calcification. Bay

scallops' mouths and digestive systems develop during their first 24 h (Belding 1910; Widman et al. 2001; Cragg 2006), which means until these are fully formed and they can feed on phytoplankton, bay scallops rely on maternally invested energy from the yolk of the egg (Le Pennec et al. 1998; Caers et al. 1999). While the quality of food on which adults feed can significantly effect larval survival and growth (Le Pennec et al. 1998; Caers et al. 1999), in these experiments, embryos were pooled and divided evenly among treatments, so any maternal effect on development should be equal across treatments. During the first day of growth, larvae experience a negative energy balance as they deplete maternally-invested reserves to fuel embryogenesis (Lu and Blake 1999). Calcification under decreased Ω (in these experiments, undersaturated Ω) is less thermodynamically favorable and more energetically costly (Orr et al. 2005; Kleypas et al. 2006). The additional energy required to build their shell in low Ω waters may limit the maximum size of bay scallop shells when larvae are faced with the finite energy reserves of the yolk, resulting in smaller shells than those of their conspecifics calcifying in ambient Ω waters. Additionally, the idea that bivalve larvae use amorphous calcium carbonate (ACC) to produce the earliest stages of their shell (Weiss et al. 2002) may help to explain the sensitivity of bay scallop larvae to high CO_2 conditions during initial calcification, as ACC is more soluble than aragonite and its formation would therefore be even less thermodynamically favorable.

Once the larvae were able to consume microalgae to increase their energy reserves, exposure to high CO_2 did not depress growth rate. This indicates that the

ration used in these experiments provides larvae with sufficient food to overcome the energetic demands of calcifying under decreased Ω. This is not surprising, as the ration was chosen because it has been shown to produce high growth rates in bay scallop larvae (Widman et al. 2001), specifically in order to ensure food limitation was not a factor in these experiments. Because the data suggest energetic limitations to growth within the first day of development, it would be useful to design experiments with varied food rations to produce food-limited and food-replete conditions, in order to see if such limitations could exist once larvae enter the planktotrophic stage.

In contrast to the timing of CO_2 exposure during initial calcification affecting larval shell size, exposure to high CO_2 during embryogenesis and the earliest larval development (prior to shell formation, 2-12 h) resulted in deformed larval shells with an abnormal indentation near the hinge (Figs. 4.3H-L, 4.4). A similar abnormal development as a result of high-CO_2 exposure has been documented once before, in 2 d old Pacific oyster (*Crassostrea gigas*) larvae exposed to water with pH 7.4 since fertilization (Kurihara et al. 2007). Light microscopy images of the oyster larvae show a dark spot near the hinge, similar to the those seen in the present study in the switch to ambient and the high-CO_2 treatments (Fig. 4.1A, C), but Kurihara et al. (2007) did not further investigate the abnormality with SEM visualization. Because this abnormal indentation occurs as a result of early exposure to high CO_2, groups who exposed bivalve larvae to high CO_2 conditions after the start of shell formation would not have observed this effect. When bay scallop embryos were exposed to

high CO_2 conditions until 2 h post-fertilization, followed by exposure to ambient conditions, larvae did not show the abnormal shell indentation (Chapter 3, Fig. 3.1), indicating that the critical exposure period for bay scallops is somewhere between 2 and 12 h post-fertilization.

At 12 h old, bay scallop larvae are generally in the late gastrula/early trochophore stage, and shell formation begins soon thereafter (Belding 1910; Sastry 1965). Shell formation begins with shell field invagination on the dorsal surface of the trochophore (Bellolio et al. 1993; Casse et al. 1998; Cragg 2006), with the shell gland located within. The earliest stage of the shell, secreted by the shell gland, is a saddle-shaped organic pellicle layer, which is not calcified yet and is essentially periostracum (Bellolio et al. 1993). The first calcified shell is called the prodissoconch I and includes a central round area with pits and radial striations, called a 'punctate-stellate pattern' (Carriker and Palmer 1979). This region overlies the embryonic shell gland and represents the initial mineralization of the organic pellicle (Carriker and Palmer 1979). The abnormal indentation appears in close proximity to the punctate-stellate region (Fig. 4.3), but based on the timing of CO_2 exposure that produced the abnormal indentation (2-12 h post-fertilization), its formation must be initiated before calcification and even before shell formation begins with the organic pellicle layer. Additionally, the abnormal indentation lies in close proximity to the location of attachment of velar-retractor muscles to the interior of the shell (Elston 1980; Cragg 2006), but these muscles are not in place until the veliger stage, which again occurs after the initiation of the indentation. A

possible explanation for the observation that exposure to high CO_2 before the start of shell formation affects later shell development is that the cells involved in calcification are disrupted by exposure to low pH early in development. In echinoderm larvae, the primary mesenchymal cells ultimately responsible for the formation of the larval skeleton begin migration to their final location during the blastula stage (Decker 1988). If a similar process occurs in bivalve larvae, it could be that exposure to high CO_2 during the blastula stage (~9 h post-fertilization; Belding 1910) disrupts the function of the cells that ultimately form the shell field and shell gland. In this case, it is likely that decreased pH, not decreased $\Omega_{aragonite}$ is responsible for the effect because calcification has not yet started at this stage in development.

Even without knowing the mechanisms by which the indentation is formed, its presence is likely to be important to larval well-being. The bivalve hinge allows the larval shell to open and close, allowing the velum to protrude, which enables the larva to swim and facilitates the intake of food particles and release of waste (Cragg 2006). An abnormality in the hinge region of the shell could prevent larvae from properly obtaining the food particles necessary for survival. While we did not see a reduced growth rate in either the switch to ambient or the high CO_2 treatments that might have been indicative of an inability to collect food, it is possible that when food is available at a lower ration than used here, an inability to collect food might be apparent. Additionally, there was a slight decline in the proportion of larvae in the switch-to-ambient and the high-CO_2 treatments with the indentation as the

larvae aged (Fig. 4.4), which could be an indication that larvae with the indentation have greater mortality than those without.

Larvae with prolonged exposure to high CO_2 conditions (the switch-to-high-CO_2 and the high-CO_2 treatments) exhibited shell dissolution of the prodissoconch I at 7 d, visible through SEM imaging (Fig. 4.3F, L). Because the high CO_2 conditions used in these experiments produced seawater undersaturated with respect to aragonite ($\Omega_{aragonite}$ = 0.63-0.76), unprotected shell is expected to dissolve. However, it should be noted that half of the larvae high-CO_2 treatment visualized by SEM did not show evidence of shell dissolution at 7 d, indicating that the periostracum may offer some form of protection to the shell from corrosive water. Dissolution was seen only in the prodissoconch I, which suggests that dissolution requires several days of exposure to undersaturated seawater before it is apparent through SEM visualization. Nonetheless, a bay scallop larva's shell is its primary defense against predators (Purcell et al. 1991; Cragg 2006) and degradation of the shell could make a larva more vulnerable to predation.

Direct effects of early high CO_2 exposure on bay scallop larval survival in the 12- and 24-h Switch Experiments are less clear than the effects of high CO_2 exposure on size, largely due to overall high mortality as well as high variability in survival among replicates within treatments. In the 12-h Switch Experiment, it is apparent that early exposure to high CO_2 negatively affected survival throughout the first week of larval development. In short-lived species such as bay scallops, survival of each larval cohort is critical to the continued survival of the adult population.

A relatively small difference in the timing of exposure to high CO_2 caused distinctly different effects on larval bay scallops, highlighting the need for a deeper understanding of the mechanisms by which CO_2 affects larval bivalve development. During the first 24 hours of development, prior to the onset of feeding, the energetic cost of calcification limited shell growth, but it is not known how changes in food availability during planktotrophic stages of development could impact growth in later larval development. Pre-calcification exposure to high CO_2 did not affect larval size, but it had a marked impact on shell structure for reasons that are still unclear. Studies of OA impacts on bivalves that focus on the earliest hours of larval development are essential, if we are to understand how early exposure to high CO_2 will affect individuals and populations.

The finding of two distinct developmental periods in which exposure to high CO_2 affects bay scallop larvae in different ways has both ecological and commercial implications. Coastal and estuarine systems where scallops live experience diurnal variability with respect to CO_2/pH conditions, with more favorable low CO_2/high pH conditions during the day and less favorable high CO_2/low pH conditions during the nighttime. Based on the findings of the 12- and 24-h Switch Experiments, there are two distinct ~12 h periods within the first day of development that affect scallop size and development. The diurnal variability in the field means that field-spawned embryos and larvae must experience unfavorable high CO_2 conditions for one of these developmental periods. In both the 12- and 24-h Switch Experiments, the shell deformity resulting from exposure to high CO_2 from 2-12 h post-fertilization

did not appear to affect larval growth or survival, so this negative effect of early high CO_2 exposure appears to be less significant than smaller shell size, which can have indirect effects on survival. Therefore, embryos spawned in the evening would experience most favorable CO_2 conditions when they are 12-24 h old, which would be advantageous for their shell growth. Additionally, the evidence that the critical periods of high CO_2 exposure are during the first day of development means that commercial hatcheries should aim to provide scallop larvae with optimal CO_2 conditions during that time. After the first day, exposure to high CO_2 does not have a major impact on larval size or shell formation, so hatcheries could limit their resource investment in monitoring and modifying water chemistry beyond the first day of development.

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Table 4.1. Mean (± SD) water chemistry for the ambient and high CO_2 conditions during the 24-h and 12-h Switch Experiments. Values for buckets represent the equilibrated water used to replace old culture water at water changes; values for culture cups represent the water in culture cups just prior to each water change; values for blank cups represent the water in culture cups with no larvae or algae added, just prior to each water change. pH, total alkalinity (A_T), temperature, and salinity were measured; all other data were calculated from pH and alkalinity using CO2SYS software (Pierrot et al. 2006) with K_1, K_2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), $KHSO_4$ from Dickson (1990), and pH measured on the seawater scale. ($[HCO_3^-]$ = bicarbonate ion concentration; $[CO_3^{2-}]$ = carbonate ion concentration; $[CO_2]$ = dissolved carbon dioxide concentration; DIC = dissolved inorganic carbon; $\Omega_{aragonite}$ = aragonite saturation state.)

Parameter	Ambient Buckets	Ambient Culture Cups	Switch to Ambient Culture Cups	Ambient Blank Cups	High CO_2 Buckets	High CO_2 Culture Cups	Switch to High CO_2 Culture Cups	High CO_2 Blank Cups
24-h Switch Experiment								
n	8	15	15	3	8	15	15	3
pH	7.975 ± 0.006	7.976 ± 0.007	7.961 ± 0.046	7.972 ± 0.010	7.320 ± 0.021	7.331 ± 0.043	7.315 ± 0.018	7.290 ± 0.004
A_T (µEq kg^{-1})	2160.2 ± 7.6	2279.8 ± 39.8	2281.3 ± 29.6	2193.0 ± 4.8	2164.2 ± 9.4	2308.6 ± 43.2	2283.0 ± 41.1	2225.7 ± 11.6
Temperature (°C)	24.3 ± 1.0	23.7 ± 0.5	23.7 ± 0.5	23.4 ± 0.6	24.3 ± 0.9	23.8 ± 0.5	23.8 ± 0.6	23.9 ± 0.6
Salinity	31.91 ± 0.14	32.19 ± 0.18	32.21 ± 0.19	32.24 ± 0.20	31.85 ± 0.17	32.58 ± 0.43	32.22 ± 0.25	32.78 ± 0.10
pCO_2 (µatm)	454.7 ± 8.7	478.8 ± 10.3	501.5 ± 69.1	464.5 ± 12.0	2417.8 ± 121.3	2502.1 ± 218.6	2566.7 ± 115.2	2649.7 ± 27.5
DIC (µmol kg^{-1})	1932.1 ± 10.2	2045.1 ± 33.4	2052.7 ± 31.4	1968.2 ± 10.3	2172.7 ± 17.2	2314.9 ± 39.5	2295.3 ± 40.6	2243.3 ± 10.0
$[HCO_3^-]$ (µmol kg^{-1})	1753.1 ± 12.7	1857.5 ± 28.0	1868.2 ± 32.7	1790.3 ± 13.8	2058.7 ± 15.3	2193.7 ± 37.8	2174.6 ± 38.5	2123.4 ± 9.8
$[CO_3^{2-}]$ (µmol kg^{-1})	165.7 ± 3.9	173.4 ± 6.0	169.6 ± 15.8	164.1 ± 4.0	43.1 ± 2.7	47.3 ± 5.4	44.6 ± 2.2	41.8 ± 1.0
$[CO_2]$ (µmol kg^{-1})	13.3 ± 0.3	14.2 ± 0.2	14.9 ± 2.2	13.9 ± 0.5	70.9 ± 4.6	73.9 ± 6.4	76.0 ± 3.2	78.0 ± 0.6
$\Omega_{aragonite}$	2.67 ± 0.08	2.78 ± 0.10	2.72 ± 0.25	2.63 ± 0.07	0.70 ± 0.05	0.76 ± 0.09	0.72 ± 0.04	0.67 ± 0.02
12-h Switch Experiment								
n	4	15	15	3	4	15	15	3
pH	7.950 ± 0.015	7.938 ± 0.076	7.966 ± 0.013	7.956 ± 0.015	7.302 ± 0.021	7.302 ± 0.036	7.289 ± 0.015	7.318 ± 0.051
A_T (µEq kg^{-1})	2082.1 ± 15.9	2137.2 ± 40.5	2149.6 ± 49.1	2117.3 ± 18.5	2070.1 ± 9.0	2097.8 ± 24.3	2100.2 ± 19.6	2085.2 ± 11.8
Temperature (°C)	24.4 ± 0.7	24.2 ± 0.2	24.2 ± 0.1	24.2 ± 0.2	24.5 ± 0.7	24.3 ± 0.1	24.2 ± 0.1	24.3 ± 0.2
Salinity	31.83 ± 0.28	32.75 ± 0.71	32.98 ± 0.74	32.43 ± 0.40	31.67 ± 0.13	32.19 ± 0.41	32.20 ± 0.33	31.95 ± 0.29
pCO_2 (µatm)	468.9 ± 21.7	504.5 ± 141.1	458.6 ± 14.1	466.5 ± 21.8	2417.0 ± 113.2	2444.9 ± 204.7	2522.9 ± 99.5	2346.4 ± 288.1
DIC (µmol kg^{-1})	1870.6 ± 19.0	1922.3 ± 50.4	1919.6 ± 37.4	1898.3 ± 22.0	2082.3 ± 10.1	2109.9 ± 29.7	2116.5 ± 21.3	2092.7 ± 25.9
$[HCO_3^-]$ (µmol kg^{-1})	1704.1 ± 21.3	1751.4 ± 59.4	1740.7 ± 29.7	1726.6 ± 23.3	1972.0 ± 8.8	1997.8 ± 26.8	2003.4 ± 19.5	1982.6 ± 21.8
$[CO_3^{2-}]$ (µmol kg^{-1})	152.8 ± 5.0	156.2 ± 19.3	165.5 ± 9.1	158.1 ± 2.0	39.8 ± 2.8	40.5 ± 3.1	39.2 ± 1.2	41.6 ± 4.1
$[CO_2]$ (µmol kg^{-1})	13.7 ± 0.7	14.7 ± 4.1	13.4 ± 0.4	13.6 ± 0.7	70.6 ± 4.6	71.5 ± 6.0	73.9 ± 2.8	68.6 ± 8.1
$\Omega_{aragonite}$	2.47 ± 0.09	2.50 ± 0.31	2.65 ± 0.14	2.54 ± 0.04	0.64 ± 0.05	0.65 ± 0.05	0.63 ± 0.02	0.67 ± 0.07

Table 4.2. Mean (± SD) size (µm) at 1, 3, and 7 d and mean (± SD) growth rate (µm d^{-1}) from 1-7 d of A*rgopecten irradians* larvae raised in four CO_2 treatment regimes; *n* = 4 replicate culture containers with measurements made on at least 15 larvae from each.

Treatment	Day 1	Day 3	Day 7	Growth Rate
24-h Switch Experiment				
Ambient	86.1 ± 3.6	95.5 ± 2.4	123.1 ± 5.2	6.2 ± 0.9
Switch to High CO_2	88.2 ± 4.4	95.0 ± 3.7	127.4 ± 5.5	6.5 ± 1.0
Switch to Ambient	71.1 ± 3.2	81.1 ± 2.4	112.9 ± 5.7	7.0 ± 0.9
High CO_2	71.2 ± 1.2	82.2 ± 4.6	117.8 ± 3.0	7.8 ± 0.6
12-h Switch Experiment				
Ambient	89.3 ± 1.3	99.9 ± 0.4	139.9 ± 5.7	8.4 ± 1.1
Switch to High CO_2	75.8 ± 0.7	93.6 ± 0.8	124.8 ± 0.6	8.2 ± 0.1
Switch to Ambient	87.4 ± 2.0	100.2 ± 0.5	132.7 ± 2.4	7.6 ± 0.6
High CO_2	73.7 ± 1.0	90.0 ± 3.1	119.4 ± 2.4	7.6 ± 0.5

Table 4.3. One-way ANOVAs of mean shell length (µm) of *Argopecten irradians* larvae from the 24-h and 12-h Switch Experiments raised in four CO_2 treatment regimes (ambient, switch to high CO_2, switch to ambient, and high CO_2) at 1, 3, and 7 d; $n = 4$.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
24-h Switch Experiment						
Day 1	Treatment	1032.425	3	344.142	31.384	<0.001
	Error	131.585	12	10.965		
Day 3	Treatment	745.645	3	248.548	24.494	<0.001
	Error	138.645	12	11.564		
Day 7	Treatment	479.765	3	159.922	6.499	0.007
	Error	295.305	12	24.609		
12-h Switch Experiment						
Day 1	Treatment	749.287	3	249.762	139.581	<0.001
	Error	21.473	12	1.789		
Day 3	Treatment	298.633	3	99.544	36.105	<0.001
	Error	33.085	12	2.757		
Day 7	Treatment	966.292	3	322.097	28.979	<0.001
	Error	133.378	12	11.115		

Table 4.4. One-way ANOVAs of proportion (arcsine-square root-transformed) of *Argopecten irradians* larvae with abnormally curved shells from the 24-h and 12-h Switch Experiments at 1, 3, and 7 d. Larvae were raised in four CO_2 treatment regimes (ambient, switch to high CO_2, switch to ambient, and high CO_2); n = 4.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
24-h Switch Experiment						
Day 1	Treatment	4.605	3	1.535	38.583	<0.001
	Error	0.477	12	0.040		
Day 3	Treatment	3.721	3	1.240	27.199	<0.001
	Error	0.547	12	0.046		
Day 7	Treatment	3.117	3	1.039	119.923	<0.001
	Error	0.104	12	0.009		
12-h Switch Experiment						
Day 1	Treatment	3.244	3	1.081	58.991	<0.001
	Error	0.220	12	0.018		
Day 3	Treatment	3.238	3	1.079	34.806	<0.001
	Error	0.372	12	0.031		
Day 7	Treatment	3.741	3	1.247	134.067	<0.001
	Error	0.112	12	0.009		

Table 4.5. One-way ANOVAs of mean percent survival (arcsine-square root-transformed) of *Argopecten irradians* larvae from the 24-h and 12-h Switch Experiments at 1,3, and 7 d. Larvae were raised in four CO_2 treatment regimes (ambient, switch to high CO_2, switch to ambient, and high CO_2); n = 5.

	Source of Variation	Type III SS	df	Mean Squares	*F*-Ratio	*p*-value
24-h Switch Experiment						
Day 1	Treatment	0.033	3	0.011	0.825	0.499
	Error	0.212	16	0.013		
Day 3	Treatment	0.010	3	0.003	1.645	0.218
	Error	0.032	16	0.002		
Day 7	Treatment	0.011	3	0.004	12.719	<0.001
	Error	0.005	16	0.000		
12-h Switch Experiment						
Day 1	Treatment	0.027	3	0.009	5.987	0.006
	Error	0.024	16	0.002		
Day 3	Treatment	0.030	3	0.010	4.032	0.026
	Error	0.040	16	0.002		
Day 7	Treatment	0.039	3	0.013	9.984	<0.001
	Error	0.021	16	0.001		

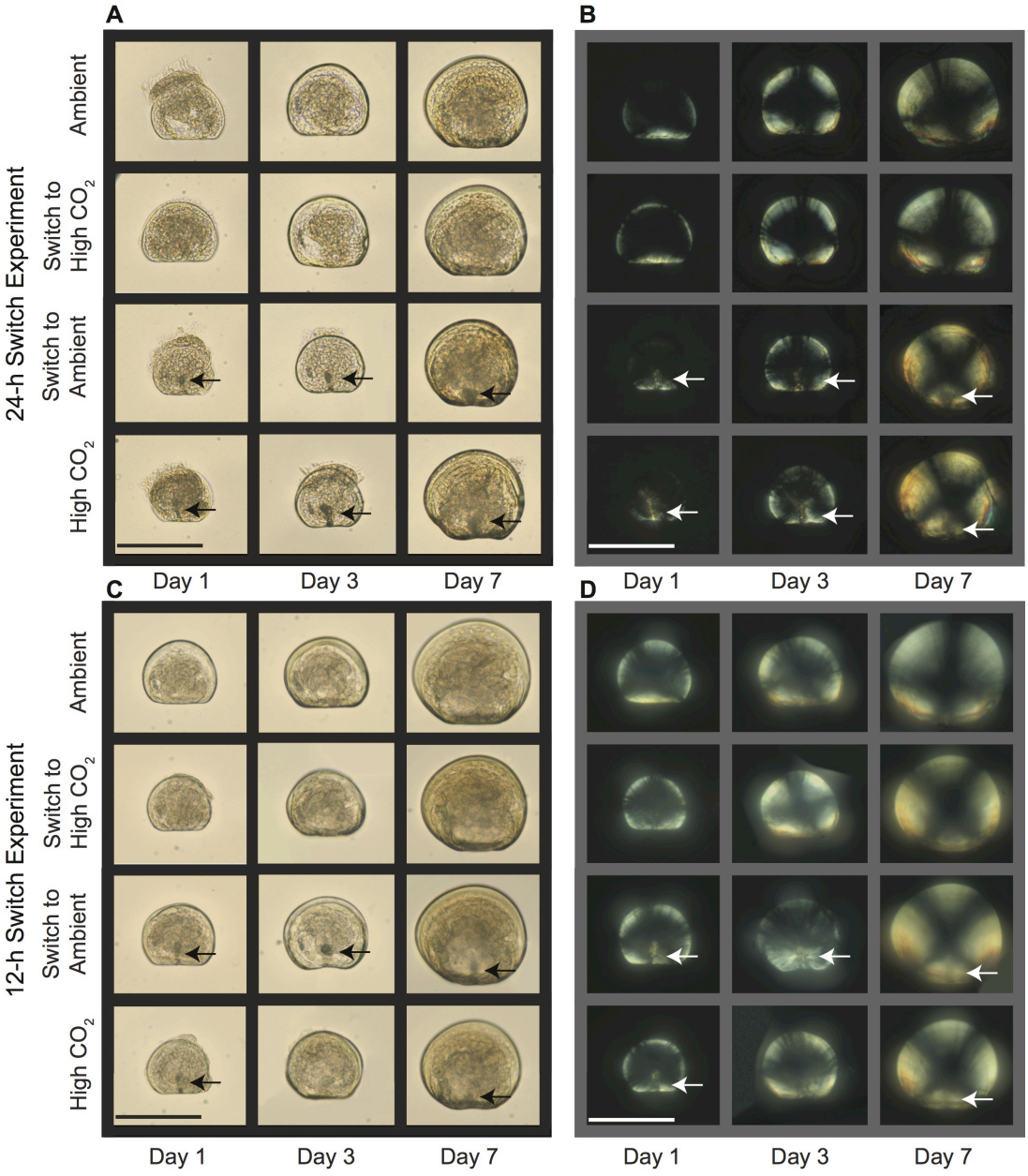

Figure 4.1. Larval morphology of bay scallops (*Argopecten irradians*) exposed to varied pCO_2 conditions from the 24-h Switch Experiment (A-B) and the 12-h Switch Experiment (C-D), viewed under both transmitted light (A, C) and cross-polarized light (B, D). Larvae were preserved in 95 % ethanol after incubation for 1, 3, or 7 d in one of the four CO_2 treatment regimes (ambient, switch-to-high-CO_2, switch-to-ambient, and high-CO_2). Larvae shown represent the mean shell length for each treatment and age. Arrows indicate the dark or light spot on the larval shell that represents a shell abnormality when visualized through transmitted or cross-polarized light, respectively. Images are all to the same scale; scale bar = 100 µm.

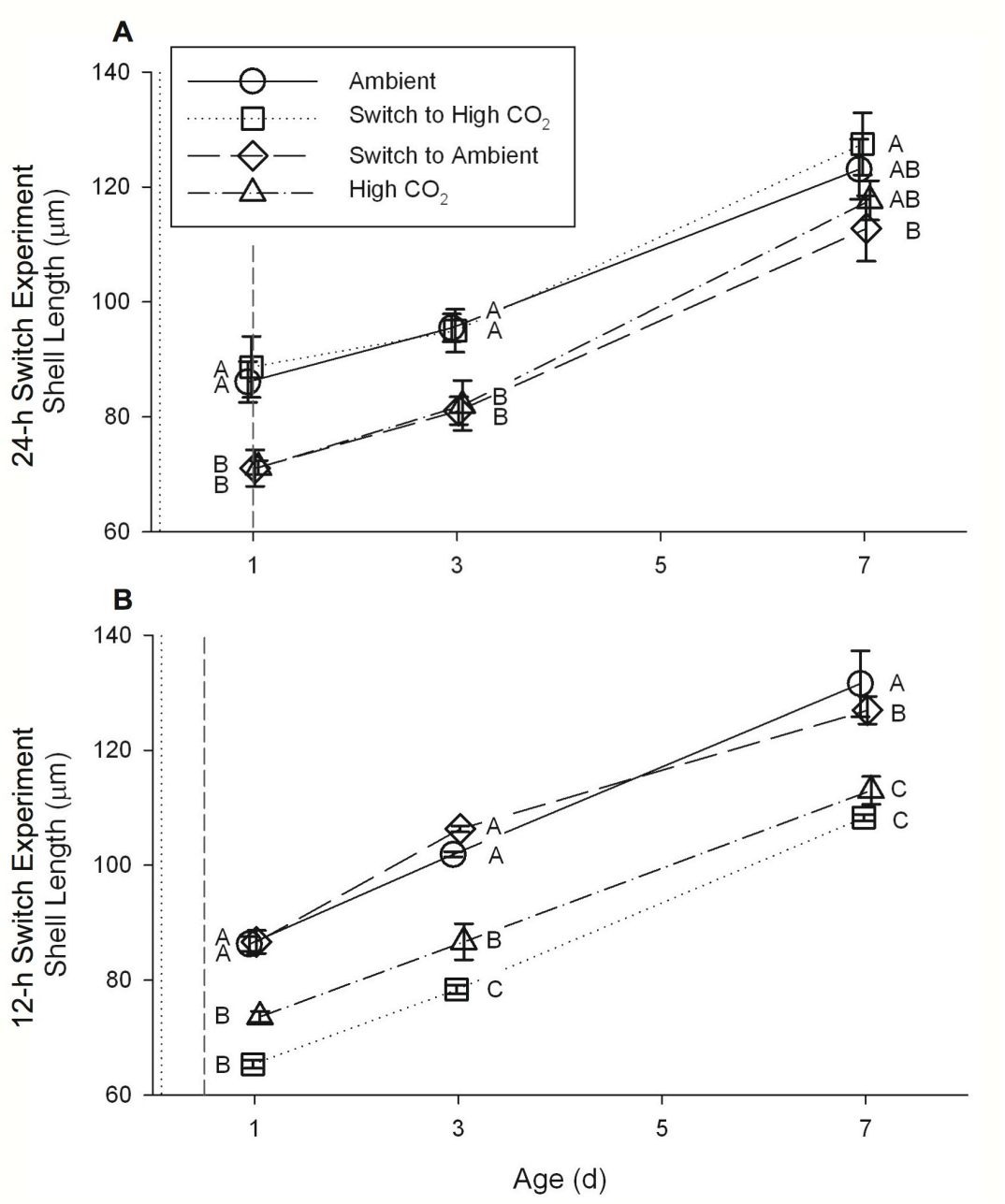

Figure 4.2. Shell length of larval bay scallops (*Argopecten irradians*) from the 24-h Switch Experiment (A) and 12-h Switch Experiment (B) during the first week of larval development. Values are mean ± SD of 4 replicate culture containers. The dotted vertical line indicates the age at the time of inoculation into culture cups; the dashed vertical line indicates the age at which CO_2 conditions were switched for the switch treatments. Different letters (A, B, C) denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 3), followed by Tukey's HSD test.

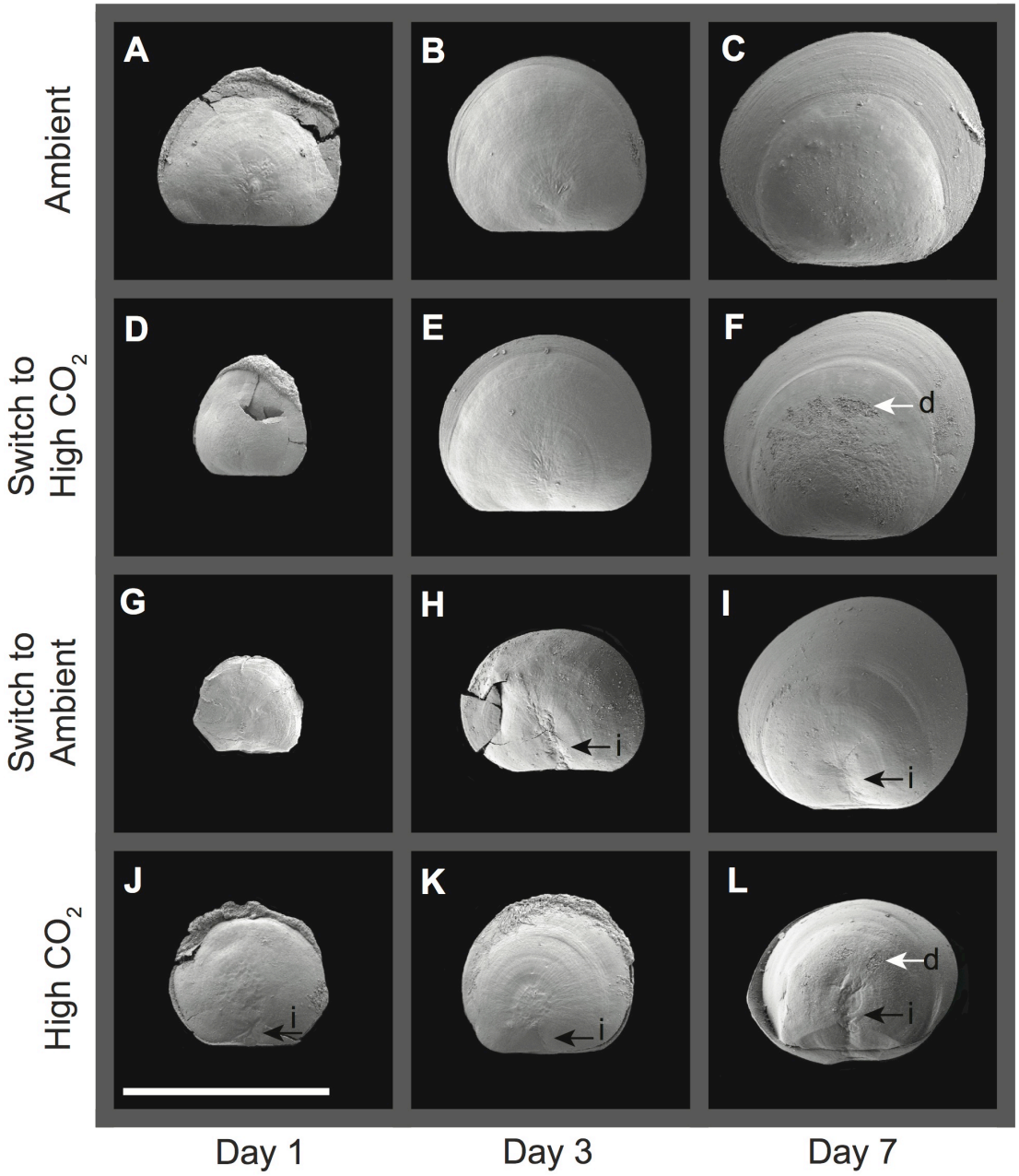

Figure 4.3. SEM images of larval shell morphology of bay scallops (*Argopecten irradians*) exposed to varied pCO_2 conditions from the 24-h Switch Experiment. Larvae shown represent the typical shell appearance for each treatment and age, but do not represent mean shell size. Black arrows point out abnormal indentations (i) near the hinges of larvae in the switch to ambient (G-I) and high CO_2 (J-L) treatments. White arrows point out dissolution (d) of the prodissoconch I on larvae in the switch to high CO_2 and high CO_2 treatments at 7 d (F, L). Images are all to the same scale; scale bar = 100 µm.

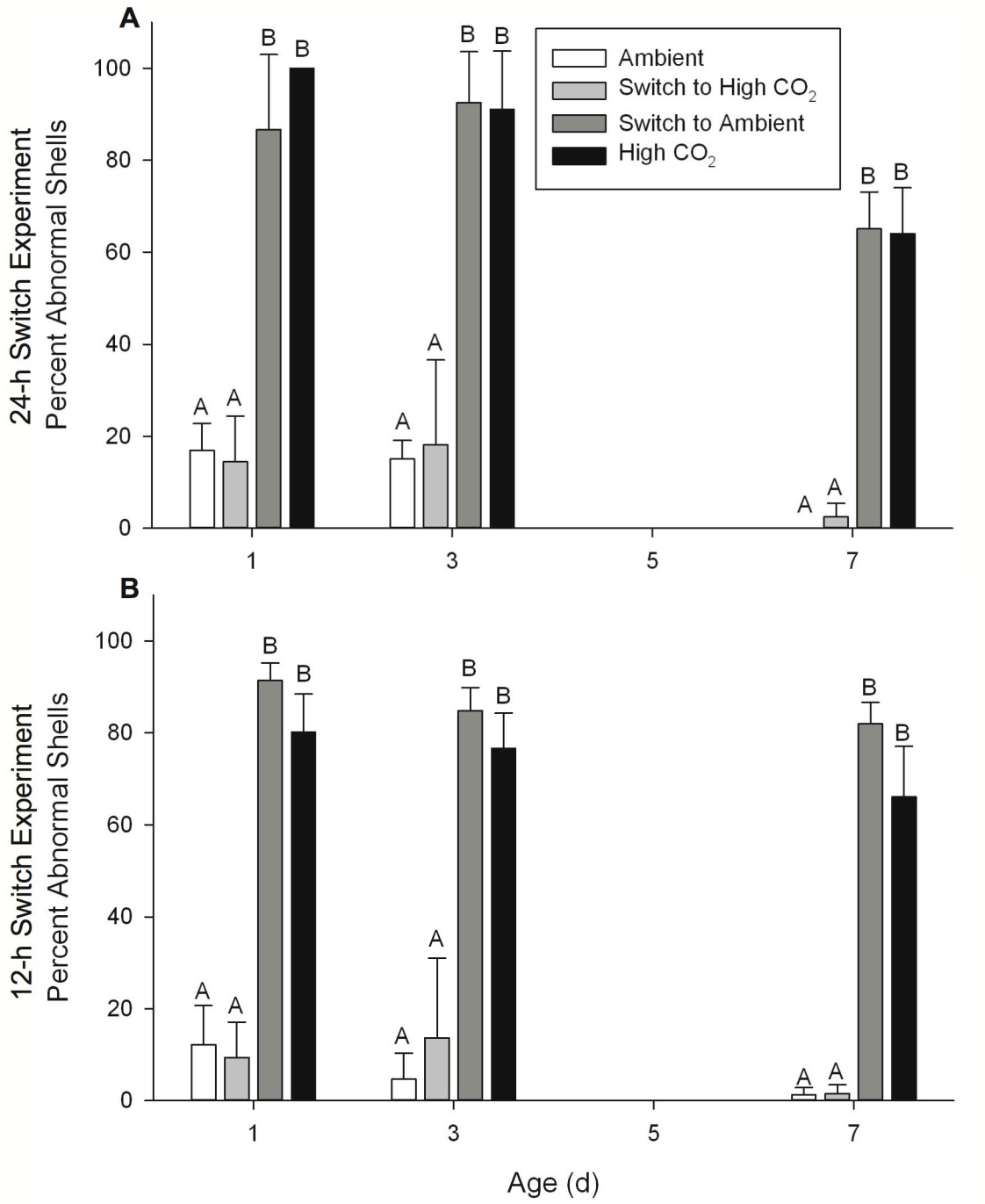

Figure 4.4. Percentage of larval bay scallops (*Argopecten irradians*) from the 24-h Switch Experiment (A) and 12-h Switch Experiment (B) exhibiting abnormal curvature of the shell near the hinge. Values are mean ± SD of 4 replicate culture containers. Different letters (A, B) denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 4), followed by Tukey's HSD test.

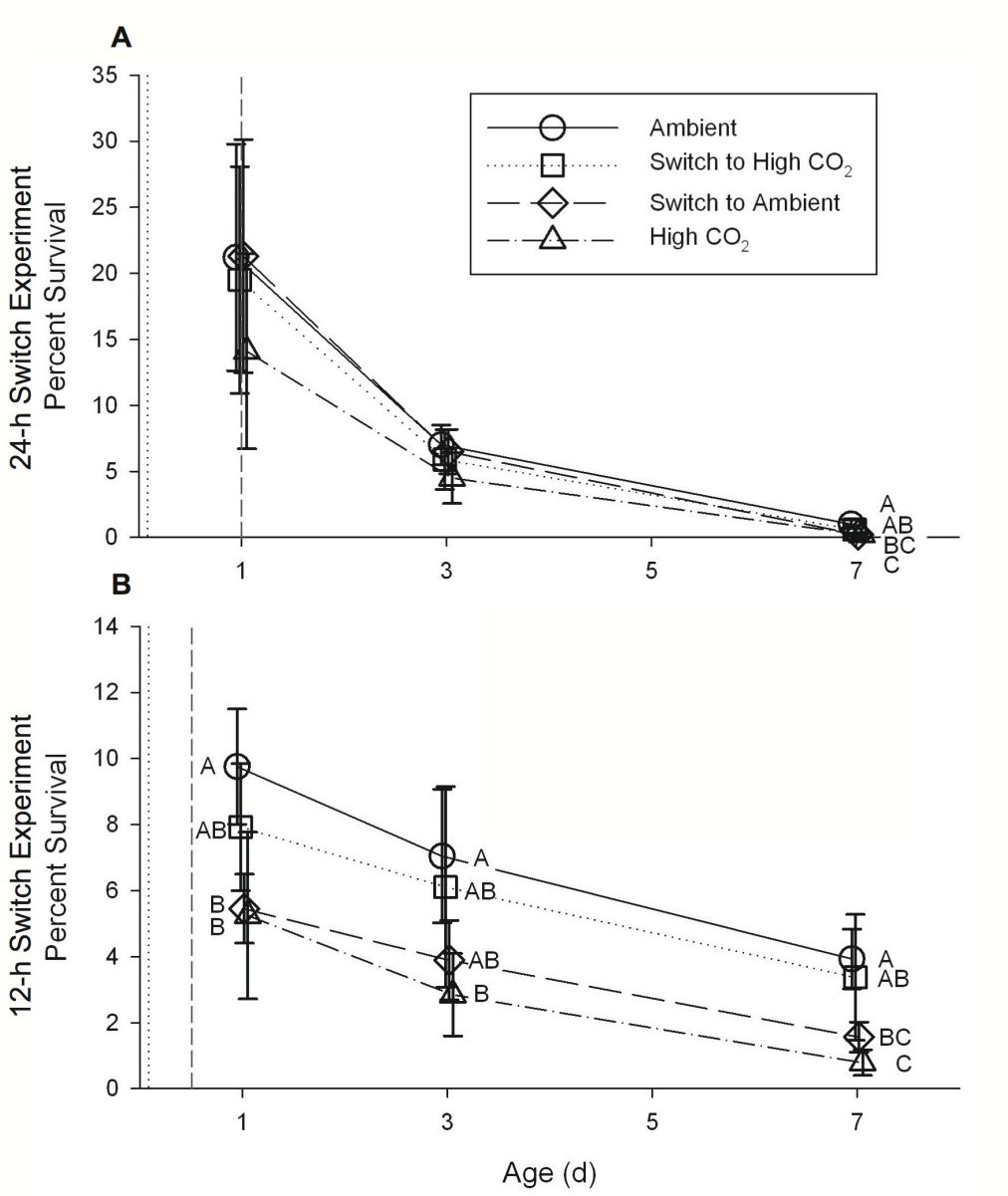

Figure 4.5. Survival of larval bay scallops (*Argopecten irradians*) from the 24-h Switch Experiment (A) and 12-h Switch Experiment (B), expressed as the percent of larvae surviving from the time of inoculation (age = 2 h), during the first week of larval development Values are mean ± SD of 5 replicate culture containers. The dotted vertical line indicates the age at the time of inoculation into culture cups; the dashed vertical line indicates the age at which CO_2 conditions were switched for the switch treatments. Different letters (A, B, C) denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 5), followed by Tukey's HSD test.

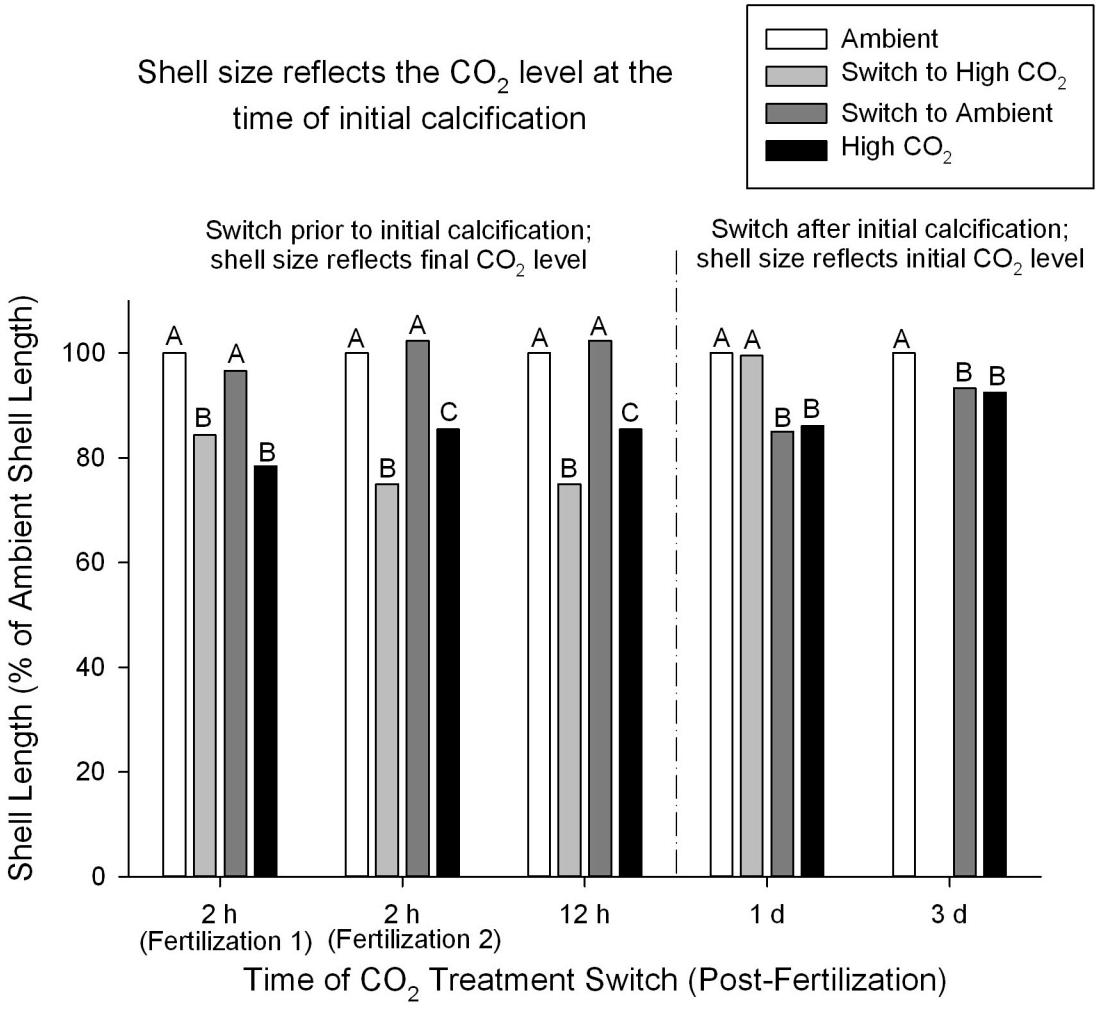

Figure 4.6. Mean shell length expressed as percentage of ambient for 3 d old larval bay scallops (*Argopecten irradians*) from five separate experiments differing in the timing of the CO_2 exposure switch. Letters above bars denote significant differences ($p < 0.05$) between treatments within a given experiment, as determined by one-way ANOVA, followed by Tukey's HSD test. High CO_2 exposure during the initial calcification period (12-24 h post-fertilization) reduces shell size, relative to ambient exposure. The trend presented here was also apparent on days 1 and 7 of all experiments.

Chapter 5

Conclusions

5.1 Overall Impacts of Early Exposure to High CO_2

I determined the stage(s) at which larval bivalves are most susceptible to damage by elevated CO_2 using a 'switch' approach, by which bay scallop larvae were exposed to ambient or high CO_2 conditions for brief periods of time. This approach allowed me to target the high CO_2 exposure to isolated stages of embryonic and larval development in order to identify critical developmental stages. Exposure to high CO_2 during initial calcification (12-24 h post-fertilization) significantly reduces shell size of larval bay scallops at 1 d, and this reduction in size is evident throughout the first week of development. Exposure to high CO_2 prior to shell formation and initial calcification (2-12 h post-fertilization) results in abnormally formed larval shells with an abnormal indentation in a dorso-ventral orientation (perpendicular to the hinge) close to the hinge. These two effects seem to be independent: exposure in the 12-24 hour window does not cause shell deformities, and exposure during the 2-12 hour window does not impact shell size. Finally, larvae exposed to high CO_2 for any amount of time had consistently higher mortality than those exposed continuously to ambient CO_2, though in these experiments the differences were not always statistically significant.

While studies to date have almost without exception shown negative effects of high CO_2 on larval and juvenile bivalves, it was unclear which early developmental stages are particularly vulnerable to high CO_2 and which stages, when exposed to high CO_2, have critical impacts on later development. Knowing this timing is important because allows us to better understand how scallops developing

in the wild will respond to changing CO_2 condtions and it also has implications for hatchery production. Furthermore, the stage-specific responses of young bay scallop to high CO_2 conditions could be used to construct a matrix population model to project how bay scallop populations will be affected by changing CO_2 conditions. I found that exposure to high CO_2 at distinctly different stages of development produces different effects (size and development) on larval bay scallops, and in particular on the earliest developmental stages, which have frequently been overlooked by previous studies.

5.2 Impacts of High CO_2 on Shell Growth and Size

The switch experiments demonstrate that exposure to high CO_2 during the period of initial calcification (12-24 h post-fertilization) is critical to larvae bay scallop size. Larvae exposed to high CO_2 during this 12-24 h period were significantly smaller, even at 1 d old, than larvae exposed to ambient CO_2 during initial calcification. When larvae were switched from high to ambient CO_2 prior to the onset of calcification (i.e. in the Fertilization and 12-h Switch Experiments), they were not significantly different in size at 1 d from larvae raised in ambient CO_2 for the first day, supporting the conclusion that it is not simply early exposure to high CO_2, but specifically exposure during the onset of calcification that impacts larval size.

While the effects of high CO_2 exposure on larval size were persistent throughout the first week of larval development, growth rate was only affected by

high CO_2 during the first day of development. The size difference persists only because once smaller, the larvae exposed to high CO_2 during calcification remained smaller, because they were growing at the same rate as larvae in the ambient pCO_2 treatment. I hypothesize that the difference in growth rate during the 12-24 hour calcification period reflects an energy constraint on larval growth. Bay scallops cannot feed during the first day and rely on maternally-invested energy reserves from the egg yolk (Le Pennec et al. 1998; Lu and Blake 1999; Widman et al. 2001; Cragg 2006). It appears that this reserve is not sufficient to meet the increased energetic demands of calcifying under high CO_2, low Ω_{ar} conditions, resulting in smaller shells when the larvae are 1 d old. After the larvae have developed mouths and become planktotrophic, by roughly 1 d old, there was no longer an effect of high CO_2 exposure on growth rate. This indicates that the ration used in these experiments (37,500 cells/mL) provides sufficient energy for larvae exposed to high CO_2 to grow at the same rate as larvae exposed to ambient CO_2. Melzner et al. (2011) found that shell maintenance of adult blue mussels (*Mytilus edulis*) is closely coupled to the energy budget; mussels on low food rations at both ambient and high CO_2 levels experienced internal shell dissolution. Additionally, work on corals has shown that when nutrients or food are replete, coral calcification rates under high CO_2 more closely approach calcification rates under ambient CO_2 than they do when nutrients or food are limited or withheld entirely (Langdon and Atkinson 2005; Cohen and Holcomb 2009; Ries et al. 2009; Holcomb et al. 2010). My experiments represent food-replete conditions; in order to more fully understand the role of food

availability on larval bivalve growth, it will be important to design experiments with varying rations of food under different CO_2 levels.

In food-limited situations (like most natural environments), it is likely that scallop growth will continue to be affected by elevated CO_2 beyond the first day of development. Evidence for this can be seen in studies by Talmage and Gobler (2010; 2011). They found that the lipid indices of 20 d juvenile bay scallops were significantly reduced with increasing pCO_2. They hypothesized that this trend was a result of inability to feed due to malformed hinges. I would propose an alternative hypothesis: that larvae are depleting lipid (energy) reserves to metabolically compensate for the higher energetic demands of calcifying shell under high CO_2, low Ω_{ar} conditions. The ration used by Talmage and Gobler was approximately half the ration than used in the present experiments and could also account for the decreased growth rate they observed in later development, a high CO_2 response that was not apparent in the results of any of my experiments. Furthermore, scallop larvae rely on the energy reserves they have accumulated during their planktotrophic phase while they undergo metamorphosis and are unable to collect food particles for a period of time (Whyte et al. 1992; Soudant et al. 1998; Lu and Blake 1999; Cragg 2006). The lower lipid indices that Talmage and Gobler (2010) observed in bay scallops exposed to high CO_2 might help explain the sudden mortality occurring around the age of metamorphosis, mortality that was higher for scallops exposed to high CO_2 than those exposed to ambient or pre-industrial CO_2 levels.

5.3 Impacts of High CO_2 on Shell Development and Morphology

The critical exposure window for larval shell deformity is the period between 2-12 h post-fertilization. Larvae exposed to high CO_2, and thus also to low pH, during this period, which is prior to shell formation and the onset of calcification, exhibited a high prevalence of an abnormal indentation near the hinge, with the deformity being noted in high frequency on all days that the larvae were examined (1, 3, and 7 d). When larvae were exposed to high CO_2 conditions after this period (all treatments in the 3-d Switch Experiment and the switch-to-high-CO_2 treatment in the 12-h and 24-h Switch Experiments), there was little or no evidence of the deformity. This indicates that exposure to high CO_2 or low pH within the first 12 h post-fertilization leads to deformity in the shell that is formed later. In the replicated Fertilization Experiments (Chapter 3), embryos that were exposed to high CO_2 only until they were 2 h old, followed by exposure to ambient conditions did not show the abnormal indentation, supporting the conclusion that the critical period of high CO_2 exposure begins after the larvae are 2 h old.

This early CO_2 exposure effect has been reported in one other bivalve species, the Pacific oyster (*Crassostrea gigas,* (Kurihara et al. 2007). This group exposed the larvae to high CO_2 (~2200 ppm), starting prior to fertilization and light micrographs of the oyster larvae show a dark spot near the hinge, similar to the those seen in the present study. However, other studies have also exposed oyster (*Saccostrea glomerata* and *C. gigas*) embryos to high CO_2 conditions during this

critical period, but have not noted deformities similar to the one found here (Parker et al. 2009; 2010). An explanation for this could be that CO_2 levels used by Parker et al. (2009; 2010) were around 1000 ppm, much lower than the CO_2 level used in the current experiments and by Kurihara et al. (2007). Perhaps there is a critical pCO_2 level at which exposure during early development produces an abnormally indented larval bivalve shell.

Prior to calcification, shell development begins with an organic protein-matrix stage similar in composition to the periostracum (Hodgson and Burke 1988; Bellolio et al. 1993; Casse et al. 1998; Cragg 2006), even the organic part of shell development initiates when the larvae are more than 12 h old. The primary mesenchymal cells involved in the calcification of the larval sea urchin skeleton begin movement to their final location during the blastula stage (Decker 1988). If a similar process occurs in bivalve larvae, it is possible that the function of the cells ultimately responsible for calcification could be disrupted by exposure to high CO_2/low pH prior to the start of shell formation, during the blastula stage (~9 h post-fertilization; Belding 1910). An understanding of the mechanisms of this effect would broaden understandings of the effects of ocean acidification (OA), high CO_2 and low pH, on marine invertebrates. Since to date, much OA research has focused calcification-related impacts, while this impact occurs prior to calcification.

A bivalve larva use the cilia on its velum to collect food particles and transport them to its mouth (Cragg 2006). In order for the velum to protrude properly, the hinge must be functional and allow the larval shell to open and close.

An abnormality in the hinge region of the shell could impair a larva's ability to obtain food and increase its risk of starvation. While there is no direct evidence for feeding impairment, it is possible that when food is limited, as in the wild, a hinge deformity might impair both growth and survival. Furthermore, a veliger larva's shell provides its most important protection from predators (Purcell et al. 1991; Carriker 1996), and any aspect which reduces the shell's integrity could cause the larva to be more susceptible to predation, which could also have indirect effects on survival.

Another OA effect that could impact shell integrity is dissolution of shells that experience prolonged exposure to high CO_2. Larvae exposed to high CO_2 for at least 6 d started to show dissolution of the prodissoconch I at 7 d. Because the high CO_2 treatment produced water undersaturated with respect to aragonite ($\Omega_{aragonite} \sim 0.65$), it is not surprising that the shell dissolved. This degradation of the shell during prolonged exposure to high CO_2 may increase the vulnerability of bay scallop larvae to predation. Because higher CO_2 levels are more prevalent in estuarine environments, scallops developing in exposed coastal areas may be at an advantage over those developing in more isolated bays.

5.4 Impacts of High CO_2 on Survival

The survival of bay scallop larvae exposed continuously to ambient CO_2 was consistently greater than that of larvae with any exposure to high CO_2. While consistent, this effect was not always significant, most likely because survival was

highly variable among replicates within treatments. With more replicate cultures for each treatment, it is possible that the survival effect would have been more pronounced.

The experiment in which the effect of CO_2 exposure was most obvious was the Fertilization 1 Experiment. While there may have been confounding maternal effects between the ambient and high CO_2 fertilization groups, it is clear that CO_2 exposure post-fertilization had a significant negative impact on survival at 1 d of larvae from the same mother. For those fertilized in ambient CO_2, this impact persisted at 3 and 7 d as well. Aside from having the most significant treatment effect on survival, the Fertilization 1 Experiment also had the highest survival at 1 d, at 47 % for those fertilized and raised in ambient CO_2. It is possible that for various reasons, the scallops providing eggs for this experiment had produced eggs with superior nutritional quality than those providing eggs for other experiments. Bivalve larvae's survival can be highly dependent on the quality of the maternally-invested energy reserves of the egg (Le Pennec et al. 1998; Lu and Blake 1999; Widman et al. 2001; Cragg 2006). The CO_2 effect in this experiment may be more pronounced than in other experiments because the larvae were initially the best provisioned, and there was therefore more of a range of mortality that could be experienced by larvae in high CO_2 treatments.

Exposure to high CO_2 generally reduced larval survival of bay scallops, but the five experiments did not highlight an obvious period in which exposure to high

CO_2 is critical to larval survival. It is possible that mortality effects of CO_2 exposure would have been more apparent in a food-limited situation.

5.5 Sensitivity of Different Life Stages to High CO_2: A Comparison of Multiple Studies

Whether investigations focus on effects of high CO_2 exposure on bivalve larval or adult life stages, researchers tend to measure similar parameters, most often including survival and growth. However, these parameters have different implications depending on which life stage they are applied to. For example, reduced shell growth of adults may affect an individual's reproductive output because gonad size is proportional to scallop shell size (Barber and Blake 2006), but it is unlikely to affect that individual's survival. In contrast, reduced shell growth of larvae can lead to delayed metamorphosis, increasing the chances of predation while the larvae are planktonic (Thorson 1950; Sastry 1965). In this case, smaller size affects the individual's survival, and, potentially, the reproductive output if the smaller size persists into adulthood. Reduced shell thickness as a result of exposure to high CO_2 would likely have similar effects on both larvae and adults, as both would become more vulnerable to predation.

Because of these nuances, it is difficult to make comparisons regarding sensitivity of different life stages to high CO_2 conditions. Such comparisons are further complicated because to date, work on different life stages has been carried out by different research groups, using different CO_2 levels and different culturing

methodologies. Additionally, many factors aside from CO_2 conditions affect survival, including (but not limited to) food ration and use of antibiotics in culturing. Nonetheless, I quantitatively compared shell growth (Fig. 5.1) and survival (Fig. 5.2) of bay scallop larvae, juveniles, and adults, using data generated by the 12- and 24-h Switch Experiments from Chapter 4 of this thesis as well as by Talmage and Gobler (2009; 2010) and (Ries et al. 2009).

5.5.1 Shell Growth of Different Life Stages in Response to High CO_2

Each group used a different measurement of shell growth: this thesis research investigated early larval growth in terms of shell length, Talmage and Gobler (2009, 2010) investigated juvenile growth in terms of both shell length and thickness, and Ries et al. (2009) investigated net calcification rate of adult bay scallops. These three different shell growth measurements show that bay scallop shell growth is negatively affected by exposure to high CO_2 conditions at all life stages (Fig. 5.1). However, it appears that the impact of high CO_2 exposure on larval shell length is not as strong as the impact of high CO_2 exposure on juvenile shell length and thickness or adult net calcification rate (Fig. 5.1). Shell length of 7 d larvae exposed to high CO_2 is larger, relative to 7 d ambient shell length, than shell length of 19 or 20 d larvae exposed to high CO_2, relative to 19 or 20 d ambient shell length (Talmage and Gobler 2009, 2010).

It is not entirely clear if this is an effect of the length of CO_2 exposure or of different culturing techniques, since different lab groups produced the two data sets.

The results of the 12- and 24-h Switch Experiments from Chapter 4 showed that larval shell size is determined by CO_2 conditions experienced during the onset of calcification and that high CO_2 exposure beyond that time period does not significantly affect growth rate. That indicates that simply a longer exposure to high CO_2 would not increase the difference in shell size between larvae and juveniles exposed to high CO_2 and those exposed to ambient CO_2. However, a major caveat of that conclusion is that it is only valid for the food ration used in these experiments. Talmage and Gobler fed their scallop larvae and juveniles nearly half the ration used in the 12- and 24-h Switch Experiments described in Chapter 4, which makes it entirely possible that growth rate could be depressed for larvae and juveniles exposed to high CO_2 beyond the 12-24 h post-fertilization period of initial calcification. Therefore, while the synthesis results (Fig. 5.1) indicate that larvae are less sensitive to high CO_2 than juveniles and adults, it could be argued that the apparent tolerance of larvae to high CO_2 conditions is a result of the higher rations the larvae were provided with, not a difference in sensitivity of larvae versus juveniles.

The three different measurements of shell growth used by different groups on post-metamorphic life stages of scallops (Fig. 5.1) show a nearly consistent negative shell growth trend with decreasing saturation state. Excluding the shell length data from 7 d larvae, and the shell length of 19 d juveniles exposed to the mid-Ω level, the remaining data from two different lab groups, representing three different types of measurements fall on a neat trend line. This indicates that

exposure to high CO_2 conditions negatively impacts shell growth, regardless of how it is measured. As discussed above, the implications of a decrease in shell diameter vary for different life stages, while a decrease in shell thickness makes any life stage more vulnerable to predation. To date, the response of bay scallop shell thickness to high CO_2 exposure has been measured only for juveniles (Talmage and Gobler 2010). Further investigations of shell thickness at different life stages would better our understanding of sensitivity of different life stages of bay scallops to high CO_2.

5.5.2 Survival of Different Life Stages in Response to High CO_2

In an attempt to identify differences in vulnerability to high CO_2 conditions across different life stages, survival of bay scallops from four different life stages were quantitatively compared (Fig. 5.2). From the work of Ries et al. (2009), exposure of adult bay scallops to a range of high CO_2 conditions for 60 d did not affect their survival. These adults were field-collected, so their CO_2 exposure earlier in life is not known, and percent survival of these adults was based on a small number of individuals (6 adults were initially exposed to each CO_2 condition). Nonetheless, the two highest CO_2 (and therefore lowest $\Omega_{calcite}$) treatments had higher survival relative to the ambient CO_2 treatment, indicating that exposure of adults to high CO_2 does not negatively impact bay scallop survival.

In contrast, survival of bay scallops that were introduced to CO_2 treatments at a young age (2 h to several days post-fertilization) showed decreasing survival with decreasing saturation state (Fig. 5.2). This pattern is seen from 7 d larvae to 38

d juveniles. It is likely that Talmage and Gobler's use of antibiotics increased the survival of larvae and juveniles in all treatments, relative to the survival rates seen in the 12- and 24-h Switch Experiments. This difference in culturing technique makes direct comparisons between lab group experiments difficult. However, comparing the results of young scallop survival from the 12- and 24-h Switch Experiments in Chapter 4, as well as from the work of Talmage and Gobler, to the results of adult scallop survival from (Ries et al. 2009), it seems that young scallops are more vulnerable to mortality from exposure to high CO_2 than are adult scallops.

The quantitative comparison of life stage-specific responses indicates that survival of early life stages is more sensitive to high CO_2, while all life stages show sensitivity with respect to shell growth. In order to translate these findings into population-level conclusions, it will be helpful to construct a matrix population model using empirically determined stage-specific responses to increasing CO_2 as parameters. Such work would allow for an understanding of how the relative contributions of direct and indirect effects on survival and reproductive output ultimately influence bay scallop population structure.

5.6 Broader Implications

The results of this thesis have both ecological and commercial implications. Coastal and estuarine regions where adult bay scallops and other bivalves live experience diurnal cycles with respect to carbonate chemistry, including pH and CO_2 (Melzner et al. 2012; NOAA 2012). These diurnal cycles are exacerbated during

summer months due to increased stratification and respiration of organic matter (Dai et al. 2006; Diaz and Rosenberg 2008; Howarth et al. 2011) and it is during this time when bay scallops spawn (Belding 1910). In a 24 h period, there are about 12 h of relatively favorable low CO_2/high pH conditions and 12 h of unfavorable high CO_2/low pH conditions. The favorable conditions generally occur in the daytime and unfavorable conditions generally occur at night (NOAA 2012). Therefore, the timing of natural spawning could play a big role in the CO_2 conditions experienced by scallop embryos and larvae.

If a population of adults were to spawn in the morning, the embryos would be exposed to favorable CO_2 conditions for the first ~12 hours of development, followed by exposure to unfavorable conditions when they are ~12-24 h post-fertilization. In contrast, embryos produced from an evening spawning event would be exposed to unfavorable conditions initially, followed by favorable conditions when they are going through shell formation and calcification. I would argue that it could be more advantageous for larvae to be exposed to favorable CO_2 conditions during initial shell formation and calcification (i.e. if the adults spawned in the evening) because this timing of exposure produces smaller shells, which can lead to indirect effects on survival. Unfortunately, little is know about the timing of bay scallop spawning in the field, other than it can be triggered by an increase in temperature, or by the presence of conspecific sperm (Belding 1910). Since increased temperature can be a spawning cue, it may be that spawning during the daytime is more likely than spawning at night, which would put larvae at a

disadvantage in terms of CO_2 exposure during the critical calcification period 12-24 h post-fertilization.

From a commercial viewpoint, many 'wild' bay scallop populations around Cape Cod and the Islands are supplemented with juveniles, or spat, raised in hatcheries. My work has shown that when larvae are exposed to ambient CO_2 during the first 24 h of development, subsequent exposure to high CO_2 does not negatively impact survival or size. This implies that hatcheries could monitor and modify water chemistry to provide bay scallop embryos and larvae with favorable CO_2 conditions during the first day of development only. This would minimize the resources and effort of water chemistry manipulation, rather than maintaining favorable conditions throughout the larval and juvenile development.

5.7 Final Conclusions

I have identified two distinct stages of development during which exposure to high CO_2 impacts bay scallop larvae,. These two effects, shell size and abnormal shell development, are distinct and are likely produced by different mechanisms. It is probable that the smaller shells are produced when larvae are exposed to high CO_2 during initial calcification because the maternally-invested energy reserves are insufficient to meet the energetic demands of calcifying in a high CO_2, low $\Omega_{araragonite}$ environment. It is possible that the shell abnormality is a result of a high CO_2/low pH disturbance of the initial organic matrix shell field formation that impacts later calcification of the shell field. These experiments provide the first evidence that

initial calcification is a critical developmental stage with respect to high CO_2 exposure, and they also provide the first evidence that exposure to high CO_2 before calcification has occurred can have latent impacts on shell development.

5.9 References

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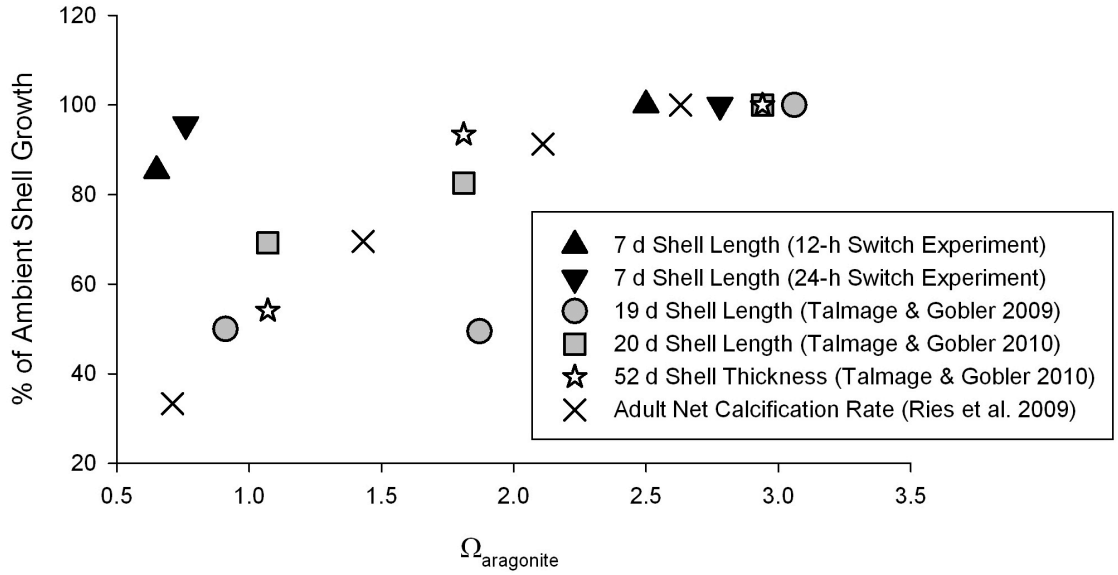

Figure 5.1. Various measurements of shell growth of the bay scallop *Argopecten irradians* exposed to a range of pCO_2 conditions resulting in varied $\Omega_{aragonite}$, expressed as % of ambient survival where 'ambient' refers to the experimental treatment with water pCO_2 closest to the current atmospheric pCO_2 value. Data for 7 d veliger larval shell length are calculated from those data presented in this thesis chapter, where embryos were exposed to treatment conditions from 2 h post-fertilization until 7 d. Data for 19 and 20 d juveniles are calculated from Talmage and Gobler (2009 and 2010, respectively) where larvae were exposed to treatment conditions starting at several d post-fertilization and lasting for the duration of the experiment. Data for shell thickness of 52 d juveniles are calculated from Talmage and Gobler (2010). Data for adult scallops are from Ries et al. (2009) and represent net calcification of adults (of unknown age) over a 60 d period of exposure to one of several pCO_2 conditions. In all cases, scallops were maintained in their respective $\Omega_{aragonite}$ conditions for the duration of each experiment with no switch in conditions (i.e., only the continuous ambient and high-CO_2 treatments from the larval experiments described in this thesis chapter are shown).

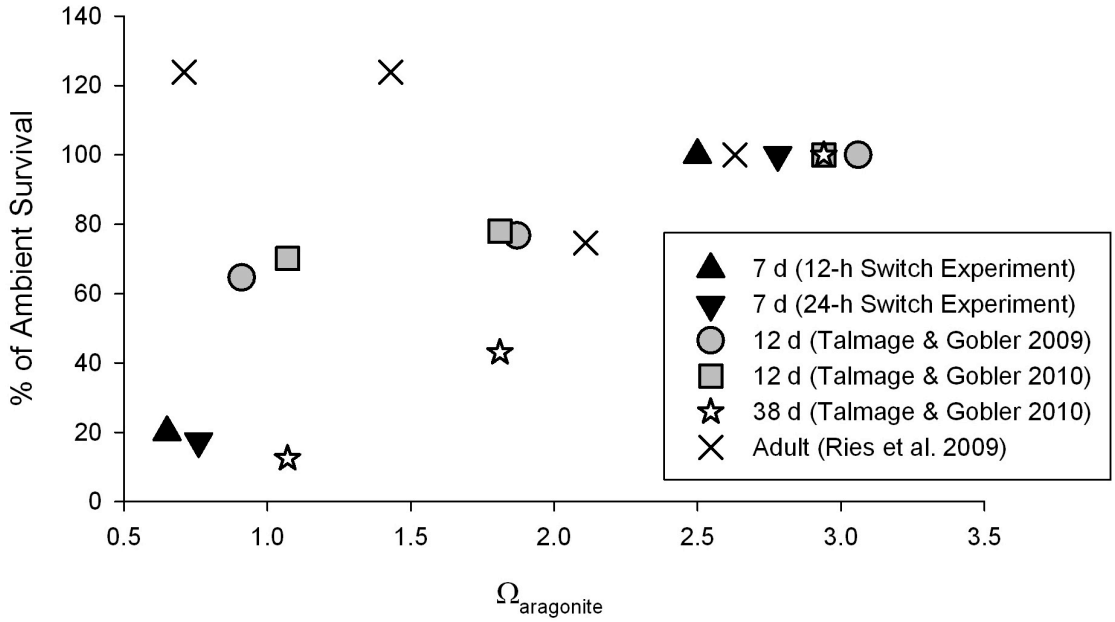

Figure 5.2. Survival of the bay scallop *Argopecten irradians* exposed to a range of pCO_2 conditions resulting in varied $\Omega_{aragonite}$, expressed as % of ambient survival where 'ambient' refers to the experimental treatment with water pCO_2 closest to the current atmospheric pCO_2 value. Data for 7 d veliger larvae are calculated from those data presented in Chapter 4 of this thesis, where embryos were exposed to treatment conditions from 2 h post-fertilization until 7 d. Data for 12 and 38 d larvae and juveniles are calculated from Talmage and Gobler (2009; 2010), where larvae were exposed to treatment conditions from several d post-fertilization until 19 d (Talmage and Gobler 2009) or 38 d (Talmage and Gobler 2010). At 12 d old, the surviving young scallops were a mix of larvae and metamorphosed juveniles. Data for adult scallops are from Ries et al. (2009) and represent survival of adults (of unknown age) exposed to one of several pCO_2 conditions over a period of 60 d. Scallops were maintained in their respective $\Omega_{aragonite}$ conditions for the duration of each experiment with no switch in conditions (i.e., only the continuous ambient and high-CO_2 treatments from the larval experiments described in this thesis chapter are shown).